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Orthotopic Implantation of Primary *N*-[4-(5-Nitro-2-furyl)-2-thiazolyl]formamide-induced Bladder Cancer in Bladder Submucosa: An Animal Model for Bladder Cancer Study[1]

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ABSTRACT

Primary bladder tumors induced in Fischer 344 inbred rats by *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide were transplanted in syngeneic rats by the intravesical, s.c., i.v., and orthotopic routes. Attempts were made to establish bladder cancer cell lines *in vitro*. No success was achieved in transplantation by either the s.c., i.v., or intravesical routes when primary tumor cells were transplanted as cell suspensions. Cell suspensions of primary tumors also failed to grow in culture. However, orthotopic implantation into the bladder submucosa gave 45% success. Tumor fragments obtained from either the primary tumor or its lung metastases resulted in 10.6 and 36% tumor takes, respectively, when implanted s.c. However, after one orthotopic passage in the bladder submucosa, the tumor cells injected as cell suspension grew s.c. in 14% and orthotopically in 79% of the animals. Tumor fragments obtained from orthotopic tumors and implanted s.c. resulted in 15% tumor takes. After the second orthotopic passage, tumor cells could be grown in cultures and orthotopically in 100% of animals. The technique of orthotopic implantation as well as the usefulness of this tumor model for bladder cancer studies are described.

INTRODUCTION

Animal models of transplantable bladder tumors in rodents are rare in spite of the fact that dyestuff intermediates are known to be bladder carcinogens in humans. Although β-naphthylamine is a known human carcinogen and 2-acetylamine fludrene, 2-methoxyl 3-amino dibenzofuran, and tryptophan are suspected in the etiology of bladder cancer in animals and humans, few transplantable bladder tumor lines were available from these studies (2–4, 9, 10, 12, 13, 18, 19). It was only in recent years that FANFT[4] was tested and shown to be a relatively specific bladder carcinogen in rats, hamsters, and mice (1, 5, 6, 7, 8, 11, 17). Soloway (15) was successful after several attempts to transplant FANFT-induced rodent bladder tumors. He showed that by traumatization of the bladder mucosa by MNU, the bladder tumor cells could be seeded in the bladder epithelium by intravesical tumor inoculation (15). The major disadvantage of this animal model, especially for the study of the intravesical immunotherapy, was that MNU caused local massive inflammation of the bladder mucosa and submucosa and infiltration of the bladder wall with acute inflammatory cells and exudate. Furthermore, our attempts to use this technique in the case of FANFT-induced rat bladder cancer in the syngeneic hosts were unsuccessful. We did not try instillation of tumor cells into bladder after its cauterization, a technique introduced by Soloway and Masters (16) that gave 54% success in tumor implantation.

In this paper, we shall describe our attempts to implant FANFT-induced tumors into appropriate recipients by several routes. Orthotopic implantation into bladder submucosa was the most convenient and dependable method for bladder cancer maintenance. In addition, the development of the transplanted tumor at the orthotopic site followed a course reminiscent of primary animal and human bladder cancer development. Our investigations have indeed yielded a bladder tumor model system ideal for the study of bladder tumor progression and testing of treatment regimens for application to human bladder cancer.

MATERIALS AND METHODS

Animals

Two hundred sixty-six inbred Fischer rats, bred in our laboratory (3 to 8 weeks old), were used as tumor recipients.

Tumors

They were FANFT-induced bladder tumors in female Fischer rats. The rats were fed FANFT for 14 months. A majority of the tumors were well-differentiated transitional-cell carcinomas. In some experiments, lung metastases of the primary bladder tumors were used. In the control group of 17 animals that received no therapy during or after FANFT feeding for 14 months, there were 13 animals with lung metastasis and 4 with suprarenal metastasis.

Routes Utilized for Transplantation of the Tumors

All the experiments described herein were carried out under aseptic conditions. The routes and modes of transplantation were as follows.

Implantation s.c. of Tumor Fragments

Tumor pieces, 8 to 15 cu mm in size, cut from the primary nonnecrotic tumor or its lung metastases, were inserted under the skin of syngeneic rats and then sutured.

Implantation of Single-Cell Suspensions

Tumor cell suspensions were prepared by mechanical tumor fragmentation with scissors in PBS. The suspended cells were removed and allowed to settle under gravity. They were washed 3 times with

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[4] The abbreviations used are: FANFT, *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide; MNU, *N*-methyl-*N*-nitrosourea; PBS, phosphate-buffered saline; i.d., intradermal.

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PBS and were tested for viability by trypan blue exclusion. The cell suspensions were inoculated by the following routes.

Subcutaneous. Five to ten million malignant cells in 0.2 ml PBS were injected s.c. in the right flank region.

Intravesical Instillation. The bladder mucosa was traumatized by MNU under Nembutal anesthesia (30 mg/kg) 48 hr before the instillation of 2 to 3 × 10^8 malignant cells/rat in 0.2 ml PBS. The tumor cell suspension was injected via a Teflon polyethylene sterile urethral catheter (19 gauge) inserted in the urethra. The fluid was retained in the bladder for 1 hr by a purse string suture around the external urethral meatus.

Injection i.v. (Transcaval). Inferior vena cava was utilized, as a vascular access, for injecting 10 × 10^6 tumor cells in 0.2 ml PBS per anesthesized rat. The time required for the injection was approximately 3 min.

Orthotopic Injection into the Bladder Submucosa. The bladder was evacuated of its urine content, and the tumor cell suspension was injected into the submucosa of a localized area of the anterior wall of the anesthesized rat with a 26 gauge 0.5-inch needle. To achieve this, the bladder was exposed and pulled upwards, gently, with a smooth forceps. The needle was then inserted obliquely for a few mm between layers of the bladder wall to deposit the cells in a submucosal location (Fig. 1). After the injection of 0.1 to 0.2 ml of tumor cell suspension, a bleb formed at the site of injection. In no instance was there breakage of the bleb and spilling of the tumor cells into the lumen. Injection of the suspension into a rat usually took about 2 to 3 min. Follow-up was done at 0.5-, 1-, and 2-month intervals to evaluate tumor growth, using a transillumination technique that permitted detection of tumors as small as 1 mm in diameter and of the development of the vascular system nourishing the tumor. When tumors were of reasonable size (0.5 to 1 cm in diameter), they were surgically removed and utilized for the second passage. In all, 14 animals were used for the second orthotopic passage.

Second-passage tumors were used in a pilot study to determine the minimum dose of tumor cells that gave 100% tumor takes. Thirty rats were divided into 5 equal groups. Each group of rats received one of the following tumor cell doses: 2 × 10^6; 1 × 10^6; 0.5 × 10^6; 25 × 10^4; and 5 × 10^4. The different tumor cell doses were injected in 0.2 ml PBS into the bladder submucosa, as described above.

An additional 50 female rats were utilized for the third orthotopic implantation. This group received injections of 25 × 10^4 tumor cells. This tumor cell dose gave 100% tumor takes in the above-mentioned study. Ten animals given injections orthotopically with the well-differentiated transitional-cell carcinoma of the bladder were followed for 6 months to study the natural history of tumor progression in this animal model of bladder cancer. These animals underwent a full autopsy when they died or were sacrificed. The bladder tumor behavior concerning growth and local invasion and metastases to lymph nodes and distant organs were studied. The rest of the animals were used in subsequent transplantation experiments.

***In Vitro* Studies.** Tissue cultures were prepared from the primary bladder tumor and from the tumors after their second orthotopic passage in the bladder.

The cultures were prepared by cutting the tumor tissue into small fragments with scissors. The fragments were seeded into plastic dishes. The fragments were cultivated under glass coverslips fixed to the dishes with silicone grease according to the method of Sly and Grubb (14). All the attempts to obtain cultures by trypsinization of tumor fragments were unsuccessful. The culture medium used was Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with 15% fetal calf serum, 100 IU penicillin, and gentamycin (100 μg/ml or 5 mg/100 ml).

For cytological examination of cell cultures, cells that had migrated from the explants into the tissue culture dishes were trypsinized, and a specimen of the cells was seeded onto microscopic coverslips in plastic Petri dishes. The cells were allowed to attach to the coverslips and form confluent monolayers during 1 to 3 days. They were then washed with PBS, fixed with methanol, and stained with May-Grenwald-Giemsa for morphological examination.

The cytological examination of solid tumors was carried out by formalin fixation of the tissue, embedding fixed tissue in paraffin, and the preparation of tissue sections (5 μm thick). The sections were fixed to glass slides and stained with hematoxylin-phloxin and periodic acid-Schiff reagents.

RESULTS

Table 1 summarizes the rate of our success in primary bladder cancer transplantation when different techniques of tumor implantation were used. The s.c. implant of a small piece of the tumor was more successful when it was obtained from the pulmonary metastases rather than from the primary tumor. Primary s.c. tumor takes were detected in 5 of 47 rats (10.6%) during the first transplantation passage and in 3 of 20 (18%) for the second passage. On the other hand, the successful tumor takes from pulmonary metastases were 5 of 14 rats (36%). They were not tried in the second passage.

A single-cell suspension from the primary tumor resulted in only one successful transplantation of 7 rats tried by the s.c. route.

No tumors developed when tumor cells were transplanted by either the intravesical instillation (with or without MNU traumatization) or the i.v. routes.

The orthotopic injection technique yielded a higher percent-

Table 1

Transplantation of FANFT-induced bladder tumors

Route of administration and the type of tumor preparation[a]	1st passage		2nd passage	
	No. of rats	Tumor takes	No. of rats	Tumor takes
I. Orthotopic, cell suspension	22	10 (45)[b]	14	11 (79)
II. s.c., cell suspension	28	0	7	1 (14)
III. Systemic (i.v.), cell suspension	9	0		
IV. Intravesical instillation, cell suspension	12	0		
V. s.c., implant of primary tumor	47	5 (10.6)	20	3 (18)
VI. s.c., implant of lung metastasis	14	5 (36)		
VII. Tissue cultures[c]	10	0		
VIII. Tissue cultures[d]	3	3		

[a] Significance (Fisher's exact probability test): first passage, II *versus* I, $p < 0.001$; III *versus* I, $p < 0.014$; IV *versus* I, $p < 0.001$; V *versus* I, $p < 0.002$; VI *versus* I, nonsignificant. Second passage, II *versus* I, $p < 0.01$; V *versus* I, $p < 0.0003$.

[b] Numbers in parentheses, percentage of tumor takes.

[c] Derived from fragments of a primary tumor.

[d] Derived from fragments of a tumor in its second orthotopic passage.

age of tumor takes. During the first passage, the bladder tumor takes were obtained in 10 of 22 rats (45%) tried, compared to 0% takes when tumor cells were injected s.c. Using the transillumination technique, the normal bladder appears semitransparent with fine vascularity (Fig. 2). The very early changes 2 weeks after orthotopic tumor implantation were in the form of neovascularity exhibited by the development of well-formed tortuous large blood vessels going toward the tumor site (Fig. 3). These vessels get larger at 4 weeks as the tumor grows (Fig. 4). When the tumors growing successfully by the orthotopic route in their first passage were converted into cell suspension and reinjected into the bladder submucosa of 14 rats, the tumor takes were obtained in 11 rats (79%).

The third orthotopic passage of the tumor in the bladder resulted in tumor takes in 100% of the rats that received the following tumor cell doses: 2×10^6; 1×10^6; 5×10^5; and 25×10^4 tumor cells/rat. It was 80% in animals that received 5×10^4 tumor cells. Using 25×10^4 cells from animals bearing orthotopic tumor in its second passage, an additional 50 rats were inoculated, and we observed 100% tumor takes for the third passage.

Using the third orthotopic passage tumor cells inoculated s.c. in a number of animals, there were 10 animals that failed to develop the tumor by the s.c. transplantation route. However, the same animals developed tumors when given injections orthotopically in the bladder (1×10^6 tumor cells/rat).

The natural history of orthotopic bladder tumor (third passage) was studied in 10 rats. The tumor developed in all the animals, reaching as big as 5 to 7 cm in diameter. No necrotic changes were found in these tumors, in spite of their large size. The tumors invaded the intestine and peritoneum locally, and bladder perforation was detected in 2 rats. Lymph node metastases were detected in all the rats. The tumor sometimes caused obstruction of the ureters with subsequent hydroureteronephrosis (Fig. 5). Lung metastases were found in all the 10 rats that survived for more than 4 months (Fig. 6). The animals, if left, died from their tumors and its distant metastases. The orthotopically injected bladder tumor cells developed into papillary transitional-cell carcinoma (Fig. 7).

DISCUSSION

Transplantable animal tumors have been a cornerstone of experimental cancer research and its subsequent application to human cancer for more than 30 years. For reasons of easy access, s.c., i.d., and i.p. sites have been preferred for tumor implantation. Although these sites are appropriate for tumor maintenance, it is questionable if the development of the tumor at these sites parallels the development of the tumor in the organ of its origin. Few experiments have been conducted on transplantation of tumors into various organs to determine favorable sites of implantation or to mimic growth of primary cancer by implantation of tumor into the tissue of its origin.

Although it was our aim to have a transplantable bladder tumor in the rat, we were not oblivious to the fact that transplantable bladder tumor growing as a primary bladder cancer would provide a more useful animal model for the study of bladder cancer therapy. Hence, intravesical and orthotopic routes for tumor implantation in the bladder were attempted along with conventional implantation routes. As described in "Results," the orthotopic route proved to be the most favorable site for transplantation of these tumors.

Our inability to grow primary tumors by the s.c. and i.v. routes is not unique. Chemically induced tumors generally require several attempts before successful transplants in syngeneic hosts are achieved. The relative ease with which the orthotopic transplant was accepted by the recipients was surprising. It is possible that implantation in the bladder submucosa and subsequent selective growth of certain primary tumor clones probably overpowered the more antigenic slow-growing tumor cells, so that future transplants were 100% acceptable orthotopically and, to a limited extent, s.c.

Histologically, the orthotopically growing tumors were transitional-cell carcinomas. From the first-passage orthotopic tumors, we were also able to obtain a cell line that grew s.c. (Table 1) when inoculated as cell suspension. This tumor proved to be squamous-cell carcinoma of the bladder. It is apparent that FANFT induces tumors of both cell types and that the s.c. milieu may be more favorable for the growth of squamous-cell carcinoma.

The technique of orthotopic implantation into the bladder is relatively simple and can be performed after opening of the abdomen as easily as a s.c. transplantation. Under aseptic conditions, mortality rate is nil. The procedure can be completed in about 3 min. Growth of the tumor can also be judged at various intervals by transillumination of the bladder without sacrifice of the animal. We believe that this model will be exceptionally suitable to study the effects of new forms of intravesical chemotherapy and immunotherapy or their combination before the application of these treatment regimens in bladder cancer patients. Partial cystectomy of the bladder to remove the tumor may also show if tumor spill into the bladder lumen during surgery results in reseeding and future recurrence of the tumor at other sites in the bladder.

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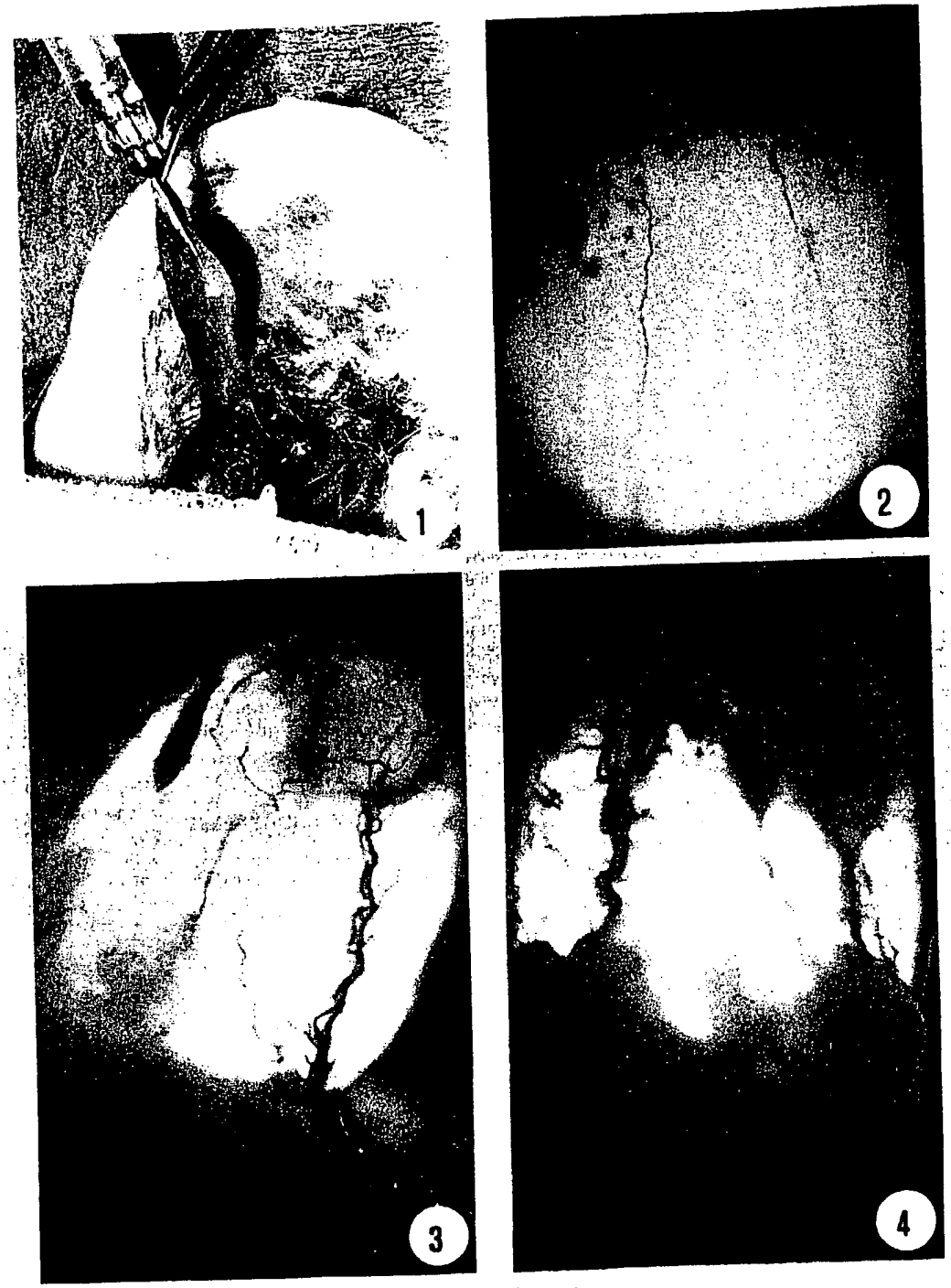

Fig. 1. Technique of orthotopic injection of bladder tumor cells into the bladder submucosa.

Fig. 2. Transilluminated photo of normal rat bladder showing fine capillaries in a semitransparent organ.

Fig. 3. Transillumination of rat bladder 2 weeks following orthotopic transplantation of bladder tumor cells. It shows the early vascular changes, which include the thickening of tortuous blood vessels growing towards the implanted tumor at the dome of the bladder.

Fig. 4. Same as Fig. 3 except that the rat bladder transillumination was carried out 4 weeks after orthotopic inoculation of tumor cells. The vascular changes have become more prominent as the tumor increased in size (see *shadow*).

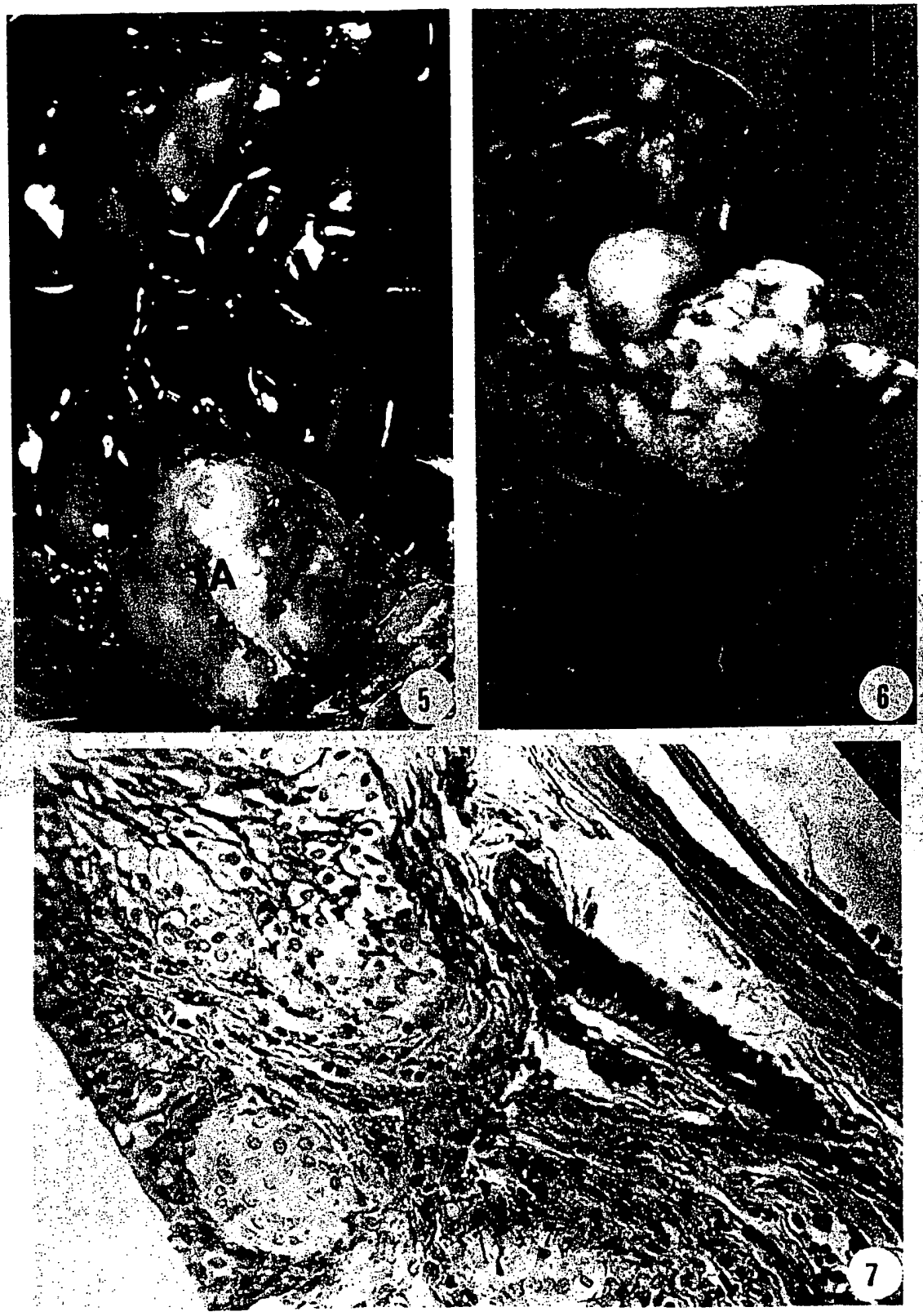

Fig. 5. A massive orthotopic bladder tumor 6 months after orthotopic inoculation of bladder tumor cells. *A*, bilateral hydroureteronephrosis; *B*, lymph node metastasis.

Fig. 6. Lung metastasis from an orthotopically implanted bladder tumor.

Fig. 7. Histological appearance of tumor in the rat bladder 10 days following orthotopic injection of 25×10^4 tumor cells. The muscle layer is free of tumor at this time, and the tumor is localized. Hematoxylin-phloxine, × 400.

14

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Chapter 20

Introduction to Techniques of Mammalian Cell Culture

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The manner in which mammalian cells are cultivated varies widely, depending not only on the requirements of the cells but also upon the applications in culture. For these reasons it is not practical to cover all aspects of mammalian cell culture in this chapter. Instead, we include only the most basic and useful procedures required to begin mammalian cell culture. In some areas, the methods commonly used are so extensive and variable that we have chosen to present them in terms of general principles. It is recommended that the novice make use of the included general references before and during initial attempts at cell culture.

20.1 COMPARISON OF TISSUE CULTURE METHODOLOGY WITH MICROBIOLOGY

Mammalian cell culture techniques were modeled after the techniques of the microbiologist. Mammalian cells, however, are more sensitive than most microorganisms to their environment. The mammalian cell is larger and is not protected by a hardy cell wall, and

its nutritional requirements are more complex. The mammalian cell in vivo is not free living as are microorganisms. The mammalian cell is dependent upon a circulatory system that has developed to ensure a precisely regulated homeostatic environment. There is an abundance of different cell types that appear to have special requirements for growth and function in vitro. Most normal animal cells will not grow (multiply) in suspension culture and require specially treated and charged surfaces for selective attachment as a monolayer and for growth. In recent years a certain amount of mystery has been dispelled about the unique requirements for consistent, controlled mammalian cell growth. Nevertheless, much remains to be learned about cell growth and differentiation.

One of the greatest difficulties in cell culture is the avoidance of bacterial contamination. The use of aseptic techniques is essential to both mammalian and bacterial cell growth, but even more stringent requirements are necessary for successful cell culture since susceptibility is greater. The introduction of a microbial contaminant into a cell culture is more devastating and more readily apparent than in a bacterial culture since animal cells grow much less rapidly than some of the common contaminants such as bacteria, yeasts, and molds. Further, freedom from chemical contamination is generally more important in cell culture than it is in microbial culture and requires that glassware be immaculately clean and that reagents be of high purity. Other physical and chemical conditions such as temperature, light, pH, and osmotic pressure must be closely monitored to provide the optimal conditions for the cells undergoing cultivation.

Early microbiologists were concerned with the problems of obtaining pure cultures, organism identification, and quantitative procedures. These obstacles were overcome with the development of solid media and differential and selective media for both the isolation and the identification of organisms. These problems also existed in the early days of cell culture, and even today they have not been solved to the same extent as they have been for microorganisms. The approaches to these problems, the success obtained, and the current limitations are presented here along with details of cell culture technology.

20.1.1 Primary Culture

Vertebrate, invertebrate, and plant cells can be grown in culture, but only mammalian cell culture is considered here. Primary culture involves the growth of cells taken directly from an animal host, put into culture, and grown for a limited time without further subpassage in culture.

Specimens

The procurement of tissue for primary cultures depends on the ultimate use of the cells. For virological studies, for example, the cells must be susceptible to the viruses. This consideration influences the selection of both the animal species and the tissue to be cultured. If a particular cell function is to be studied, then the selection of tissue may be more critical than the selection of the animal species. The fact that host cells from some species are more readily cultivated than those from other species is well known. A review of the literature generally provides information to assist the investigator in the proper selection of host and tissue for a particular area of study.

Sterility

There are a variety of techniques used to initiate cell cultures, but of primary concern is the need for sterility. Internal organs, glands, and tissues of the host generally are sterile in vivo, but they must be removed and processed aseptically to provide usable cultures. The use of antibiotics in the culture medium may add assurance, but strict aseptic technique is still essential. At times the tissue to be cultivated may come from a host tissue that is not normally sterile, such as the skin, oral cavity, or gut. In this case the area from which the tissue sample is taken should be cleaned as thoroughly as possible and well rinsed with sterile balanced salt solution. A solution of 70% ethanol may be used on the skin provided it is completely rinsed away before dissection. Mercurials should not be used since they are extremely toxic to cells in culture and cannot be adequately removed by rinsing. In some cases it may be advantageous to treat the host with antibiotics for several days before tissue excision; if so, antibiotics must be included in the culture medium during the period of cultivation. Penicillin and streptomycin at 100 U and 100 μg per ml of medium, respectively, are commonly used. Gentamicin may be used alone or in combination with penicillin. A 50-μg/ml dose of gentamicin is generally adequate, but the concentration can be increased severalfold if necessary. In addition to these antibacterial antibiotics, it may be necessary to include antifungal compounds such as amphotericin B or nystatin. These are much more toxic than the other reagents and must be used with care. If germfree animals are available, such procedures can be eliminated. There is always the possibility that the tissue, whether or not it comes from normally sterile tissue, will contain latent viruses or be infected with mycoplasma. Little can be done to control these types of contaminants. Latent viruses can present a safety hazard because they may grow to a high titer in the cultured cells (21). All primary cell cultures, as well as the materials and reagents coming into contact with them, should be handled as if they were infectious.

20.1.2 Cell Strains

The primary cultures discussed above will often contain multiple cell types. These cultures generally are used as primary cultures and then discarded. There are, however, many established cell strains or lines, some of which have been purified by cloning to provide cultures of a single cell type. This type of culture is maintained by serial passage much like cultures of a microorganism. There are two general varieties: (i) the established or continuous line, which can be grown indefinitely in serial passage, and (ii) the finite life-span strain, which undergoes a limited number of population doublings in vitro before growth stops and the cells become senescent. The latter is

usually diploid (2*N* normal chromosome numbers), whereas most established cell lines vary in their ploidy. The finite life-span strains are generally limited in their split ratios to low numbers, while many of the established cell lines can be split at ratios of 1:10, 1:20, or greater. A split ratio of 1:10 is produced by subculturing the cells from 1 flask into 10 flasks. Details of the preparation and handling of both primary and cell line cultures are presented below.

20.2 BASIC LABORATORY REQUIREMENTS

20.2.1 Sterile Work Area

Maintenance of sterility in the work area is essential for both short-term and long-term cultures. A sterile room environment is achieved by using high-efficiency particulate air filters under positive pressure so that all of the air is flowing out of the room. When cost and space are of concern, laminar-flow hoods can be used to provide a sterile work area. In some instances, Plexiglas boxes or inflatable germfree isolation chambers are suitable. The use of stainless-steel tables and work surfaces is recommended since they can be more readily disinfected and kept clean. Various arrangements can be made for storing needed supplies within convenient reach as in the microbiological laboratory. Equipment should never be stored or stocked inside the laminar-flow hoods since this interrupts the laminar flow.

In addition to providing a sterile environment for the protection of the cultures, consideration must be given to safeguarding laboratory workers when there is a risk of exposure to known or potential pathogenic agents. Therefore, precautions must be taken, especially when working with human materials. The work area should be thoroughly cleaned and disinfected before and after each use (see reference 3).

20.2.2 Equipment Washing and Preparation, Basic Equipment, and Storage Facilities

In the preparation area, separate dedicated sinks should be available for cleaning glassware and other tissue culture equipment. There are a number of glassware-washing machines currently available that minimize the handling of glassware and so reduce breakage. Sonication baths are also available that appear to be satisfactory in removing even the most stubborn material. Since all glassware and equipment should be scrupulously clean, a source of highly purified water should be available. Some laboratories routinely use triple glass-distilled water, while others depend on ultrafiltration systems. Ultrafiltration systems have been identified as sources of microbial and chemical contamination of water due to growth on filters and resins. Arrangements should be made for periodically checking the purity of the water by measuring both inorganic and organic content.

Proper care of cell culture glassware begins in the laboratory. All equipment should be rinsed with water as soon as possible after use to prevent drying of materials on the glassware. Equipment to be autoclaved should be filled with or submerged in water before being placed in the autoclave.

A glassware cleaning procedure that has worked well for us is described below.

Cleaning glass culture vessels

1. Remove all markings (grease pen or ink) on glassware before the glass is soaked or simmered.
2. Do not allow the glassware to dry between use and the cleaning procedure. (Glassware should be submerged or filled with water immediately after use as previously noted.)
3. Place the glassware in a stainless-steel bucket containing distilled water (12 liters) and 56 g of Calgolac (Calgon Corp., St. Louis, Mo.).
4. Simmer for 1 to 2 h. Be sure no air bubbles are trapped in the glassware. (The use of large sonicators eliminates this requirement.)
5. Remove glassware from the cleaning solution, and rinse in tap water five times and then in glass-distilled water five times.
6. Rinse three times in triple glass-distilled water.
7. Invert, allow to drain dry, plug with gauze-wrapped cotton, and wrap with Patapar paper (James River Corp., Edgly, Pa.). Do not use aluminum foil or brown paper. The foil is coated with lubricant that volatizes during sterilization and condenses on the glassware. Brown paper contains formaldehyde and other chemicals that are toxic.
8. Sterilize glassware in an oven at 135 to 150°C for 2.5 to 3 h.

Cleaning glass pipettes

1. Let pipettes stand in cleaning solution (distilled water and Calgolac as described above) overnight.
2. Rinse in tap water 10 times.
3. Rinse in triple-distilled water 10 times.
4. Drain dry, plug, and wrap with Patapar paper.
5. Sterilize in oven as described above.

It is desirable to dry all equipment thoroughly before sterilization; therefore large drying racks in a clean area are required.

Sterilization of equipment requires a dry-heat oven. The oven may require a mechanism for circulating air to avoid hot and cold spots in the oven, depending upon the load and the capacity of the oven. Items that cannot be sterilized by dry heat may have to be autoclaved. This can be a source of difficulty since the steam used in many systems contains volatile compounds that may deposit on the equipment.

Storage facilities

Media and sera require storage at either −20 or 4°C. There seems to be a wide variation in the storage lives of different media and sera. The length of storage life may be related to the sensitivity of the cells and to the selection of cells permissive to certain environments. In general, several recommendations can be made. Serum should be stored at the lowest available temperature, frozen and thawed as infrequently as possible, and shielded from visible light. Samples of serum sufficient for weekly use can be thawed and subsequently stored at 4°C until used.

Some media can be stored at −20°C, although the solubility of some of the ingredients is minimal at this

TABLE 1. Basal Eagle medium-Earle salts

Component	Concn (mg/liter)	Component	Concn (mg/liter)
Amino acids		Inorganic salts	
L-Arginine	17.40	$CaCl_2$ (anhydrous)	200
L-Cysteine	12.00	KCl 400.00	400
L-Glutamine	292.00	$MgSO_4 \cdot 7H_2O$	200
L-Histidine	8.00	NaCl	6,300
L-Isoleucine	26.00	$NaHCO_3$	2,200
L-Leucine	26.00	$NaH_2PO_4 \cdot H_2O$	140
L-Lysine	29.20		
L-Methionine	7.50	Vitamins	
L-Phenylalanine	16.50	Biotin	1.00
L-Threonine	24.00	Calcium-D-pantothenate	1.00
L-Tryptophan	4.00	Choline chloride	1.00
L-Tyrosine	18.00	Folic acid	1.00
L-Valine	23.50	i-Inositol	2.00
		Nicotinamide	1.00
Other components		Pyridoxal · HCl	1.00
D-Glucose	1,000.00	Riboflavin	0.10
Phenol red	10.00	Thiamine · HCl	1.00

temperature. If the precipitate does not redissolve upon thawing, sonication in a water bath may resolve this difficulty. Storage at 4°C results in the depletion of some of the nutritional ingredients due to chemical instability. Glutamine, for example, has a half-life of approximately 30 days at 4°C in solution.

Incubators

Different conditions of incubation may be required for different cells. Thus, it may be necessary to have more than one incubator of the same type adjusted to the appropriate temperature and CO_2 concentration. Incubators include both standard incubators used in closed culture without provision for gassing and CO_2 incubators for cultures that are open to the atmosphere. There are many commercial suppliers of the various types of incubators.

For large-scale cultures a warm room can be constructed, although modular units can be purchased commercially. It is important to have circulating air in these incubators to avoid hot and cold spots.

20.2.3 Glassware and Plasticware

Most items commonly found in a microbiology laboratory are standard items in the cell culture laboratory. Many of these items are now made of disposable plastic. Plastic petri dishes commonly used in microbiology laboratories are not suitable for attachment-dependent cell culture. Special treatment of the plastic surface is required to promote cell attachment and growth. The specific items needed vary depending on the research being conducted and the preferences of the investigator. Polystyrene has been accepted as the standard substrate for disposable culture vessels. The surface must be treated to provide a wettable or charged surface. This is usually done by the manufacturer. Other plastics such as polyvinyl chloride, polycarbonate, polytetrafluoroethylene, and others have been used in special situations. Polystyrene is probably the least expensive, and it is optically clear. One pitfall of plastic labware is that plasticizers can leach out during storage or use, sometimes resulting in cell toxicity and variation in cell growth and function. Plastic vessels ordinarily should not be reused.

Glass culture vessels. Reusable glass culture vessels have been used since the early days of cell culture. The fact that glass can be reused with good, consistent growth-supporting properties if properly cleaned and sterilized is a distinct advantage. The reuse of glassware often requires additional support people depending on the glassware volumes required.

20.3 CULTURE MEDIA

The medium is probably the most important factor in the maintenance and growth of cells. There are no universal guidelines for the selection of medium, serum, or growth factors for a given cell type. For most continuous cell lines it is recommended that the cells be kept in the medium to which they are accustomed. Cells may be adapted to other media by proportionally reducing the concentration of the original medium while increasing the concentration of the new medium at each passage. Growth curves can be done for each set of variables, determining lag time, generation time, and cell density at the stationary growth phase. Since there are numerous media used in mammalian cell culture, it is recommended that the novice refer to more complete descriptions before a selection is made. The ingredients are chosen to provide maintenance or growth of the cells. An example of a typical minimal medium is shown in Table 1.

Osmotic pressure

The medium environment must satisfy more than just the nutritional requirements of cells. Proper osmotic pressure is one parameter that must be met. The osmotic pressure is maintained by the appropriate concentrations of salts and glucose. Cells from different species vary in their ionic requirement. Sodium/potassium ratios in sera from various species

are known to differ, and this should be considered when selecting culture media.

Hydrogen ion concentration

The pH of most biological fluids is maintained near neutrality. Deviations of more than several tenths of a pH unit from normal are usually fatal. Most cells survive at a pH range between 6.8 and 7.6; however, many functions of cells vary with small changes in pH. Cell attachment to substrate is generally enhanced by a pH of ~7.0 or less. pH in most media is controlled by a combination of dissolved gases (sodium bicarbonate buffer system) and products of metabolism by the cell (especially lactic acid). The bicarbonate system is the natural buffer in blood.

The chemical reaction of the sodium bicarbonate buffer system is as follows: $NaHCO_3 + H_2O \rightarrow Na^+ + HCO_3^- + H_2O \rightarrow Na^+ + H_2CO_3 + OH^- \rightarrow Na^+ + OH^- + H_2O + CO_2$. Thus, one of the final products of the reaction is gaseous CO_2. The solubility of CO_2 decreases as temperature rises, causing the CO_2 to be released from the medium. This results in an increase in pH. For this reason cultures must be tightly stoppered and in some cases charged with CO_2 gas to prevent the loss of CO_2 and the rise in pH. (This point is critical during membrane filtration of media under a vacuum.) An alternative procedure is to incubate cultures in an incubator with a CO_2-enhanced atmosphere with the caps loose. The amount of CO_2 gas required to maintain stable pH depends on the concentration of $NaHCO_3$ in the medium. CO_2 incubators must be used for the incubation of cells in petri dishes unless special media without the bicarbonate system are used (24). Another consideration is the pK_a of sodium bicarbonate (6.3 at 37°C), which results in less than optimum buffering at the normal culture pH of 7.2 to 7.4.

Other buffers have been developed to substitute for the bicarbonate buffer. One of these (2) utilizes the buffering capacity of the free bases of the amino acids and the higher concentration of phosphate buffers. Another buffer used extensively for incubation in open systems is HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), which acts as a dipolar ion. A concentration of 25 mM or less is commonly used. For some applications a combination of the sodium bicarbonate system and HEPES can be used. Additional details of pH control and measurement can be found elsewhere (3). It has been demonstrated that some cells have a requirement for CO_2 (30).

Carbohydrates

A source of carbohydrate is necessary in cell culture medium to supply an energy source. The most common source of carbohydrate is glucose, but other monosaccharides (e.g., galactose) (24) can also be used. (Disaccharides, however, cannot be used.)

Amino acids

Amino acids are required for cell growth and function. Most animal cells have a specific requirement for 13 amino acids: arginine, cysteine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine. Only the L forms are utilized, but some older medium formulations contain DL-amino acids.

Vitamins

Vitamins are used as cofactors in cell metabolic functions. In certain cell types, such as bone cells, ascorbic acid is important for growth and function (11). Vitamin E has recently been shown to protect against the damaging effects of light (14). Vitamins can be provided by the serum, but with a reduced serum concentration vitamins must be provided by supplementation.

Trace elements (minerals)

Most minerals required by cells in culture are provided by the serum. In low serum concentrations (25) or in the complete absence of serum, requirements for iron, copper, zinc, selenium, molybdenum, and other minerals have been demonstrated. The levels of minerals in serum can be influenced by mineral levels in the soil, which affect the animal through its food chain. Thus, selenium levels vary in animal serum depending on the geographic location. Since high levels of these minerals are toxic to cells (14), knowing their concentration in serum and making appropriate adjustments in the medium are important.

20.4 SERA, SERUM-FREE MEDIA, AND GROWTH FACTORS

20.4.1 Sera

Serum contains growth factors and growth inhibitors. Most cell lines require the supplementation of medium with serum, but there are many cell lines that have been adapted to serum-free growth (e.g., HeLa, LLC PK_1, and L929). Although some cell selection may have been involved in addition to adaptation, recent experiments by Peehl and Ham (26) demonstrated that epidermal keratinocytes can be selectively grown from an inoculum that contains fibroblasts by the addition of selective growth factors in serum-free medium.

The sera most frequently used in tissue culture are fetal bovine serum, bovine (calf) serum, equine serum, and human serum. Proteins are a major component of serum. The functions of proteins are not well defined, but they may serve as carriers for minerals, fatty acids, and hormones or as growth factors or hormones themselves. A number of peptides in serum have been identified as growth factors and have been purified for use as supplements in serum-free medium (9). As with media, there are no universal guidelines for the selection of sera for a specific cell type. Generally, trial and error methods are employed to select the serum best suited for the growth and function of cells. Consideration must also be given to lot-to-lot variation. Suppliers will often send samples for testing with specific cell systems and will reserve these lots until they receive the results of stringent testing. Tests on sera should include sterility testing (including mycoplasma), growth curves, cloning efficiency, and microscopic observation of the morphological characteris-

TABLE 2. Growth factors for optimization of serum-free media

Growth factors
Epithelial cell growth factor
Fibroblast growth factor
Nerve cell growth factor
Platelet-derived growth factor
Transforming growth factor
T-cell growth factor
Colony stimulating factor
Triiodothyronine
Hydrocortisone
Selenium
Estradiol
Prostaglandins
Insulin
Prolactin
Growth hormone
Somatomedin
Transferrin
Hemin

tics of the cells. All or none of these tests may be applied as needed. The methodology for growth curves and cloning efficiency is described below.

Problems encountered with serum

Endotoxins. The effects of bacterial endotoxins on cells in culture are numerous. For instance, endotoxins are known to affect macrophage function and migration, stimulate hormone secretion, affect hormone sensitivity, receptor affinity, and hemopoietic cell proliferation, etc. High levels of endotoxins are usually introduced by bacterial contamination during serum collection and processing; therefore, sera should be obtained from a supplier that performs quality control assays routinely.

Hemoglobin. The hemoglobin content of sera can be used as an indicator of the quality of the procedures used in collecting serum. Hemin affects cell differentiation, induces globin mRNA, promotes neurite outgrowth in nerve cells, is a macrophage-dependent T-cell growth factor, and is mitogenic for human peripheral blood mononuclear cells. It is therefore advisable to use serum that has a low hemoglobin content unless these features are desired.

Lipids and fatty acids. Certain lipids and fatty acids are known to affect cell growth and function (10). Fatty acids, in particular, influence cell function because they are an essential and major component of cell membranes and they are determinants of membrane fluidity. It is important to recognize these features since many cell characteristics, including cellular enzyme activity, growth rates, virus survival, and infectivity, can be altered by varying the concentrations of fatty acids in serum.

The lipid requirement for the nutrition of cells remains controversial and has not been systematically studied. Recently, Bettger and Ham (10) demonstrated the preferential delivery of lipids to cells via liposomes in contrast to the established perference for serum lipoproteins. Many cell lines show a growth response to supplemented lipid when serum-supplied lipids are limiting or absent. Requirements for linoleic acid, other fatty acids, cholesterol or cholesterol precursors, the fat-soluble vitamins, and phospholipids have been demonstrated in cell culture.

Hormones. Cell culture plays a major role in delineating hormone activities (9). It is therefore important to recognize the value of identifying and controlling the levels of hormones in serum before setting up experiments to study the effects of particular hormones on cell function.

20.4.2 Serum-Free Media

Optimization of serum-free media is a time-consuming process with no general rules to follow and so must be carried out by trial and error (9). Some supplements, such as insulin, transferrin, and selenium, are commonly used. Other supplements are more cell specific, including estrogens, androgens, triiodothyronine, and others (Table 2).

During the development of serum-free media for well-established cell lines, optimization can be accomplished one step at a time. This may not be practical when establishing primary cultures, since the variability of primary cultures is inherent. The work of Barnes and Sato (9) has clearly demonstrated the ability of complex mixtures of hormones, growth factors, and carrier proteins to replace the functions of serum. In culturing human keratinocytes, Peehl and Ham (26) demonstrated that ethanolamine can be substituted for pituitary extract. By increasing the concentration of iron and decreasing the concentration of zinc, the need for transferrin in keratinocyte culture was eliminated. These examples illustrate the complexity of growth requirements.

20.4.3 Growth Factors

The most commonly used growth factors are listed in Table 2. Obviously, this list is not complete since growth factors for specific cell types are the attention of current research.

Substrate

A proper substratum is essential for the growth of some differentiated cells, especially in defined medium. Studies have demonstrated that plastic is not an optimal substratum. Coating plastic with substances from the extracellular matrix such as fibronectin, chondronectin, laminin, and collagen has been shown to have clear advantages in the attachment and growth of cells.

Defined media

Defined media are important for the discovery of novel substances with growth-promoting activities (9). High-density lipoproteins are essential for the growth of some kinds of cells. Cholera toxins, glucagon, thrombin, vitamin E, prolactin, and small proteins (growth factors) are also required by cells. Studies with defined media have helped to identify growth factors and growth inhibitors in serum.

20.5 METHODS FOR PRIMARY CULTURE

20.5.1 Explants

The oldest procedure for setting up primary cultures is the explant method. In this method, the tissue is washed free of blood and cut into small fragments of about 2 mm^3. The best cutting method is the use of two scalpels held with the blades opposing each other and manipulated like a pair of scissors. The fragments are rinsed well with balanced salt solution or culture medium and held in a small volume of either. The fragments must be placed in a culture vessel and anchored to the growth surface. Chicken plasma (rooster) prepared with heparin is commonly used to embed and to anchor the explants. After the explants are in place, the plasma can be clotted by the addition of a few drops of chicken embryo extract. Some tissues such as testicular explants cause coagulation of the plasma without any additive. When the plasma is firmly clotted, an appropriate volume of liquid culture medium is added as an overlay, and the cultures are incubated at 35 to 37°C. Within a few days new areas of cell growth or migration are seen (microscopically) around the edge of the explant. The cultures should be refed two to three times per week during the growth period.

20.5.2 Enzyme-Dispersed Cells

The preparation of cultures by enzymatic digestion of minced tissue was introduced in the early 1950s as a means of readily preparing large numbers of primary cultures for viral propagation and assay. This method was originally applied to kidney tissue, on which it perhaps works best, but has been found useful for other tissues as well. Trypsin (0.25%) is commonly used, but for some tissues collagenase is more effective. The tissue is minced (barber's shears work well for this), washed to remove blood and debris, and suspended in the enzyme solution. The general procedure involves some form of agitation over time, with harvest of the loosened or free single cells by filtration through a gauze filter or similar device. The coarser, larger fragments retained by the filter are subjected to additional cycles of enzymatic digestion followed by similar harvests. The harvested cells are pooled and held in ice water until the process is completed. The cells are counted with a hemacytometer, and a suspension of a desired density is prepared for planting into culture. (There are a variety of more specific procedures for the enzymatic digestion of tissue which are described elsewhere [2].) The suspended cells (after planting in a stationary vessel) settle, adhere to the culture surface, and replicate. Over a period of time with intermittent medium renewals, the cells form a monolayer of growth over the entire available area and appear to be much like the cultures of cell lines described in the following section. It is important to realize that trypsin is most active at an alkaline pH and that the enzyme solution should be kept at pH 7.6 to 8.0. A typical procedure for preparing rabbit kidney cells is given below.

Preparation of primary rabbit kidney cells

1. Use 21-day-old rabbits.
2. Aseptically remove kidneys and place them in chilled growth medium (medium 199) containing 1.5% $NaHCO_3$ and 5% equine serum or fetal bovine serum.
3. Place kidneys in a petri dish, remove capsule and medulla, and rinse well with medium.
4. Transfer kidneys to mincing tube and mince to fine particles (~0.5 to 1.0 mm).
5. Rinse with 125 ml of warm 0.25% trypsin per kidney. Shake gently until tissue is no longer clumped, and then decant the trypsin.
6. Add 125 ml (per kidney) of warm trypsin and a magnetic stirring bar, and place over magnetic stirring motor for 15 min at speeds below those producing foam.
7. Filter the cell suspension through three layers of sterile gauze into a 250-ml centrifuge tube in an ice bath.
8. Repeat step 7 until no large pieces remain.
9. Centrifuge the filtered cell suspension at 250 × g for 10 min.
10. Remove the trypsin supernatant fluid by aspiration.
11. Resuspend the cells in 200 ml of the above medium. Centrifuge as described above.
12. Filter through gauze and repeat step 11.
13. Resuspend cells in 20 ml of medium and transfer into two 15-ml graduated tubes. Centrifuge at 250 × g for 5 min.
14. Dilute the cells to 1:400 in the above medium on the basis of a volume of packed cells.
15. Disperse in culture flasks (4 to 5 ml/25-cm^2 flask).

20.5.3 Blood and Ascites Cells

In a few instances, cells can be obtained directly from the host as a suspension of cells. A pleural effusion or ascites will provide such cells, as will the peripheral bloodstream. These ascites may be induced in an animal by the inoculation of an irritant into the peritoneal cavity; the ascites can then be used to collect macrophages for culture. Cells can be removed from their suspending medium by centrifugation or by selective adsorption to and elution from appropriate materials before being placed in a culture medium and planted in culture.

Primary cultivation of leukocytes from peripheral blood has become a common practice for immunologists. Of the cellular elements in blood, generally only the lymphocytes and monocytes can be induced to proliferate in culture. Methods for separating these cells into reasonably pure suspension have been developed (27). Monocytes and lymphocytes may be separated from the other elements by density gradient centrifugation. The lymphocytes and monocytes collect at the interface, while erythrocytes and polymorphonuclear leukocytes settle below the interface. Monocytes may be further separated by adherence to filters of glass beads or nylon columns. If the monocytes are to be retained for cultivation, adherence to petri dishes or other plastic culture flasks can be utilized. The remaining lymphocyte population is composed of T cells (about 75 to 80%), B cells (1 to 15%), and a minor but important population of null cells. The lymphocytes collected after one of the above procedures can be further subdivided by adsorption to sheep erythrocytes, layered over Ficoll-Hypaque, and

centrifuged. The B cells collect at the interface, whereas the T cells attached to the erythrocytes are found in the lower level of the tube. Most null cells are contained in the B-cell fraction but some are also with the T cells. The T cells are freed from the erythrocytes by lysing the latter with NH_4Cl-Tris or by osmotic shock with distilled water. Further purification of the B cells-null cells can be accomplished by taking advantage of the immunoglobulin on the surface of B cells. An antibody prepared against the $F(ab')_2$ portion of the immunoglobulin of the species from which the cells are obtained will react with the surface immunoglobulin. This antibody may be adsorbed to a Sephadex G-200 column for this purpose. The B cells when added to the column are retained, while the null cells are collected in the effluent. B cells can be recovered by treating the column with immunoglobulin of the species under study (27).

Purified suspensions of lymphocytes do not grow in culture unless induced by mitogens, cytokines, transforming agents (such as viruses), or other cellular elements which provide the necessary growth factors. Macrophages or monocytes are commonly used as helper cells. T-cell growth factor, leukocyte activating factor, and B-cell growth factor are examples of cytokines that are used. Two common mitogens that can be used are phytohemagglutinin and concanavalin A. Epstein-Barr virus may be used for human cells if a transformed population is desired.

Cell suspensions for cultivation can be obtained by mechanical methods from some soft tissues like thymus or spleen. This is done by trituration, stirring, or even forceful pipetting. With such methods the tissue cells must be freed of all blood elements by washing and centrifugation. It may be necessary in some instances to treat the cell suspension with NH_4Cl or by osmotic shock to remove contaminating erythrocytes. The resulting cell suspension is planted in culture where, depending on cell type(s), the cells will either settle, adhere to the growth surface of the flask and proliferate, or else remain in suspension during their growth period.

20.6 CELL STRAINS

20.6.1 Monolayers

Most cell strains grow attached to glass or plastic surfaces. Numerous types of culture vessels are available. The adherent cells can be subcultured by resuspending the cells in fresh medium and dividing into new vessels. Several methods available for detaching adherent cells are as follows. (i) Proteolytic enzymes, most commonly trypsin (crude) and pancreatin (also crude), can be used. Other enzymes that are used are collagenase, elastase, hyaluronidase, pepsin, pronase, etc. The choice of the enzyme depends on cell sensitivity and on the processes for stopping the action of the enzyme. (ii) Chelating agents such as EDTA are used to resuspend some attached cells. (iii) Combinations of enzyme and EDTA are frequently employed. (iv) Physical methods, e.g., scraping from the glass with a rubber policeman and shaking, can sometimes be used to loosen cells. These techniques are not recommended for cells that adhere tenaciously to the culture vessel surface. Some of these cells can be suspended as single cells by gentle pipetting.

A stepwise procedure for suspending a monolayer culture of cells is described below.

1. Prepare a sterile solution of trypsin (1:250; Difco Laboratories, Detroit, Mich.) at a concentration of 0.1 to 0.5% in medium or balanced salt solution at pH 7.6 to 8.0. (More dilute solutions are used for cultures in serum-free medium since natural trypsin inhibitors are absent.)
2. Decant or aspirate medium from the culture. Since serum inhibits trypsin activity, it may be necessary to rinse the cell sheet with serum-free medium.
3. Add enough trypsin solution to cover the cell sheet.
4. The time needed for trypsin treatment varies with cell type, but 1 to 5 min is usually required. Incubate at either room temperature or 37°C. The process is complete when the cell sheet appears to be loosened from the surface.
5. Add sufficient medium to make a uniform suspension. Then aspirate the cells in small-bore pipettes to finally disaggregate the cells.
6. Centrifuge the suspension as slowly as possible to pellet cells and minimize gravitational forces.
7. Resuspend the cells in an appropriate volume of complete medium. If cell dilution is required for subculturing, make appropriate dilutions as needed.

Cold trypsinization results in less damage to cells. Again, the sensitivity of cells to trypsin is the factor to be considered in deciding whether to use 4, 25, or 37°C for treatment. The cells can be suspended in trypsin solution or in complete medium with serum. In serum-free medium, a trypsin inhibitor can be added. However, since crude trypsin contains other enzymes, inhibition of enzyme activity is not complete.

Alternatively, cells can be resuspended as follows. After enzyme solution is added to the flask, incubation is carried out for 1 to 3 min (longer for more firmly attached cells), and the flask is inverted, allowing most of the enzyme solution to drain away from the cells. Digestion is allowed to proceed to the point at which small constrictions can be seen in the cell sheet upon microscopic observation. At this time, the trypsin is removed, and culture medium is added. The cells can be resuspended by gentle pipetting in complete medium and then inoculated into new culture flasks.

Note: Low temperatures are known to cause disaggregation of microtubules, rendering the cells more susceptible to damage from shearing forces produced by pipetting or other physical treatments.

Seeding density

The cell concentration for inoculating fresh vessels is usually about 100,000 cells per ml of medium or 20,000/cm^2 of growth area. The cell concentration is usually determined by the growth rate of the cells, which in turn is a function of the cell type, the medium, and the environment. Most cells have a finite density for both seeding and maximum density.

20.6.2 Suspension Culture, Stationary Suspension Culture, Soft Agar Culture, and Agitated Microcarrier Suspension Culture

As with bacteria, the easiest and simplest way to grow mammalian cells is in suspension culture. Most cells of leukemic or lymphoid origin grow as single cells in either stationary or agitated suspension cultures.

There are many apparatus for growing cells in suspension culture. Cherry and Hull (16) devised suitable vessels from round-bottom flasks which were agitated by a Teflon-coated magnetic stirring bar suspended from stainless-steel swivels by stainless-steel wire. The flasks were then mounted at a suitable distance from a motor-driven magnet to reduce the effect of heat generated by the motor.

Large-scale suspension cultures of mammalian cells have recently been described by Feder and Tolbert (17). Spin filter techniques and flexible plastic sheets designed to rotate at slow speeds are used to retain cells in suspension without developing excessive shear forces that are disruptive of cell membranes. Many variations of these techniques have been designed, including continuous perfusion.

The gas phase requirements of suspension cultures are variable. Some investigators simply sparge with air; others use mixtures of air and CO_2 or O_2. The generation of oxygen bubbles in the medium should be avoided.

Agitated microcarrier suspension cultures

Many anchorage-dependent cells will not grow in suspension culture unless special procedures are used. One of these procedures involves the use of Sephadex beads to which the cells attach. These beads can then be kept in suspension by adequate agitation techniques. Various additives such as 1% methylcellulose or polyethylene glycol can be added to the medium to prevent serum precipitation on the sides of the vessel. Other modifications of the medium can be made such as the reduction of calcium or phosphate to reduce cell clumping. There is general disagreement concerning the benefits of these modifications that is probably related to particular cells and culture conditions. For these reasons, many variations of the method have been devised. Microcarrier beads are made of natural or synthetic polymers of various sizes, such as Sephadex, dextran, etc. A problem encountered with the beads involves collisions which damage the attached cells. If cells need to be harvested, it is often difficult to remove them from the beads by conventional techniques. A variety of bead types is available from commercial suppliers.

Modified perfusion systems have also been used with the microcarrier system. Of particular significance is the microcarrier-perfusion reactor described by Feder and Tolbert (17). Through better control of the environment, cell densities have been obtained that are 5 to 10 times greater than those previously attained. An additional feature of this procedure is an efficient scale-up system that minimizes the lag times usually encountered in such systems.

Soft agar cultures

Semisolid and soft agar techniques have been developed since some malignant or virus-transformed mammalian cells can grow into isolated colonies in highly purified soft agar culture. Double-strength medium is used to suspend cells, and the cell concentration is adjusted to twice that of the desired final concentration. Highly purified agar (agarose) is made up in triple-distilled water at concentrations ranging from 0.6 to 1.0%. The agar is sterilized by autoclaving, brought to 41 to 44°C, and held in a water bath. The medium containing the cells is warmed to 35°C and added to an equal volume of warm agar. The medium and agar are quickly mixed, transferred into petri dishes, and incubated in CO_2 incubators. Colonies can be counted or isolated for cloning purposes. A modification of this technique is the human tumor stem cell assay developed by Hamburger and Salmon (20). In this method a feeder layer of nutrients is provided beneath the agar layer containing the cells.

20.6.3 Hollow-Fiber Systems and Other Substrata

During the last 10 years, hollow-fiber culture equipment has been the subject of much developmental effort, especially for mass cell culture techniques. Typically, in this system a cluster of porous hollow fibers is enclosed in a vessel. The ends of the fibers are arranged so that the medium or the gaseous environment, or both, can be continually changed. The cells are implanted on the outside of the fibers, creating a large surface area for cell growth. Cells can grow to high tissuelike densities and can be maintained for months.

20.7 MAINTENANCE OF CELL CULTURES

20.7.1 Subculturing

Once a culture is established, whether it is a cell line or a primary culture, it requires periodic medium change or subculturing. Intervals between medium change and subculturing are determined by rate of cell growth, depletion of nutrients from the medium, accumulation of metabolites in the medium, and pH change.

Conditions to consider when deciding to subculture or refeed the cultures are an increase in cell concentration, a drop in medium pH, and the microscopic appearance of the cells. Generally, normal cells stop dividing when they reach a stage of confluency, when essential ingredients are depleted from the medium, or when a toxic metabolite builds up in the medium. Cells that are not dividing may still deplete some nutrients from the medium. Careful and timely assessment of the cultures is crucial to the establishment of proliferation and function.

20.7.2 Clones and Cloning

In mixed cell cultures, the fastest growing cell type will eventually dominate. To select and propagate specific cell types, a number of different cloning procedures have been developed. These include limited dilution cloning in a variety of conditions and media

designed to select a single cell, or the progeny of a single cell, for further growth. Since single-cell growth is a function of plating efficiency and medium volume, optimization of the medium plays a critical role in successful cloning (23). A number of procedures have been devised for achieving this goal such as cloning in soft agar, the use of cloning rings in monolayer culture, selective irradiation, selective media, interaction with substrate, cell migration, and single-cell sorting by flow cytometry. (See references 2 and 7 for detailed procedures.)

20.8 TYPES OF CELLS CULTIVATED

The use by earlier workers of the plasma clot and media with high concentrations of serum and embryo extract favored the growth of fibroblasts or the connective tissue elements from the tissues put into culture. Most tissues or organs contain connective tissue in the form of capsules and internal structural or binding materials which generally have a greater propensity for growth in vitro than do the more differentiated parenchymal cells. Subcutaneous or skin muscle tissue, especially from embryos, provides a good source of fibroblasts for culture preparation. Other, more specialized, connective tissues such as cardiac or skeletal muscle, bone, cartilage, and adipose tissue and the supporting elements of the central nervous system such as neuroglial cells, can be grown in culture.

20.8.1 Cardiac and Skeletal Muscle Cells

Embryonic or neonatal hearts provide the best opportunity for culture of myocardial cells (13). These can be cultured as explants or as trypsinized cell suspensions; and, in the proper environment, contracting cells develop and can be maintained in a beating state for fairly long periods of time. When enzyme-dispersed cells are used, contaminating endothelial cells can be eliminated by taking advantage of their quickness to settle and adhere to glass or plastic surfaces. With skeletal muscle, only the mononuclear myoblasts proliferate in culture; but as the cell density increases, cell fusion occurs to produce multinucleated muscle fibers which no longer divide. After fusion, contraction of the multinucleated fibers may be seen, and the typical cross-striation of skeletal muscle cells develops (31).

20.8.2 Bone Cells

Cultures of bone cells (osteoblasts and osteocytes) are usually initiated from embryonic bone or primordia, but one report of the long-term cultivation of adult bone cells has appeared (28). Embryonic calvaria provide a good source of cells, which can be obtained from both the periosteum (endocranial surface) and the bone itself (11). The tissues are separated mechanically, washed, minced, and trypsinized to disperse the tissue into a suspension of single cells for planting into culture. A medium referred to as BGJb or its modification, BGJb/FJ, plus fetal bovine serum (10 to 20%) is recommended for the growth of these cells. The cells settle, attach to the floor of the culture vessel, and proliferate, becoming confluent in about 7 to 14 days. As growth continues the cultures become multilayered, and dense areas of cell growth are observed. Mineral (Ca^{++}) deposition occurs and is most evident in the areas of high cell density. Cultures can be initiated from explants of embryonic bone in which cells migrate out from the explant and proliferate. Other bones such as ribs or femurs have been used as a cell source. In addition to the cell culture methods described above, some truly remarkable studies have been done with organ cultures of rudimentary bone in which development of the organ was demonstrated.

20.8.3 Chondrocytes

Chondrocytes from elastic, hyaline, and articular cartilage (obtained from the ear, sternum, and long bone articular surfaces) can be grown in cell culture (19). Following excision of the tissues, all adhering noncartilaginous tissue is dissected away, and the clear cartilage is cut into small fragments (1 to 2 mm^3). These fragments are dissociated by incubation in a medium of 0.5% collagenase–10% fetal bovine serum in salt solution with mild or no agitation. The suspended cells are collected, washed by centrifugation, and suspended in growth medium. Ham F12 medium (2) with 5% fetal bovine serum is satisfactory. After being planted in culture, the cells settle, attach to the growth surface of the culture vessel, and proliferate to form colonies of epithelial-like cells. Some elongated fibroblastlike cells may be seen which represent contaminating cells from connective tissue not completely removed during the preparation of the cartilage for cultivation. Chondrocytes can be subcultured from the primary cultures by using trypsin, but long-term or continuous cultures of these cells are not available.

20.8.4 Adipocytes (Fat Cells)

Adult fat cells do not proliferate, but cultures of adipocytes can be prepared from precursor cells obtained from adult fatty tissue. The tissue is treated with collagenase in a buffer solution and agitated over time at 37°C. The time of digestion can be determined by microscopic observation of the tissue and cells. The use of siliconized glassware is recommended. After incubation the tissue remnants are removed by filtration of the cell suspension through a nylon screen into a centrifuge tube. The fat cells float to the surface and can be harvested for planting into culture. Medium 199 (12, 29) with a serum supplement, plus such additives as insulin, methylcellulose, increased glucose, and linoleic acid, has been used to cultivate the cells. The cells settle, adhere to the culture vessel, spread out, and become elongated like fibroblasts. As growth continues a monolayer is formed, and at this time granules of lipid-staining material appear in the cytoplasm. With continued incubation the granules or lipid droplets coalesce into a single large lipid deposit, and the cells begin to appear as adult fat cells. These will loosen from the growth surface of the flask and float freely in the medium. After harvest, the floating lipid-laden cells can again be treated with collagenase and returned to culture where the process will be repeated (12, 29).

20.8.5 Nerve Cells

Tissue culture had its origin in the experiments of Harrison (2) with nerve tissue maintained in vitro. In the ensuing years, many investigators have studied cells and tissues of both the central and peripheral nervous systems in culture systems. Neurons from brain and spinal cord ganglia can be maintained in culture where, with optimal conditions, maturation with growth of fibers and axons can be observed. Cell replication does not occur unless neuroblasts from very early embryos are used. Cells of the supporting neuroglial tissue, however, do replicate, and continuous cell lines of glial cells have been developed. Pieces of brain tissue are carefully dissected to remove all membranes and extraneous material and then cut into small pieces, put into medium, and dissociated by vigorous pipetting. The resulting suspension of cells and small aggregates is plated into culture vessels where some cells will slowly settle, adhere, and replicate to form monolayers of glial cells. Several weeks may be required for this to occur. Cells can be subcultured by the trypsin method and serially passed for a limited number of cell generations. Eagle medium or Ham F-10 (2) with fetal bovine serum is satisfactory.

20.8.6 Glandular Epithelial and Vascular Endothelial Cells

Epithelial cells, once thought to be difficult to grow in culture, are readily cultivated from a number of tissues by using media and culture procedures now in common use. The use of either lower concentrations of serum in defined media or serum-free media with added growth factors has made growth of these cells possible. The most readily cultivated epithelial cells are those lining organs or tubules or those from the skin. After careful dissection to remove connective tissue elements, the tissue is minced and dissociated with trypsin or collagenase to prepare cell suspensions for planting into culture. In some instances an organ or tubule might be filled or perfused with the enzyme solution to loosen or free the epithelial lining cells. Kidney tubules may provide the best source of epithelial cells for culture by these methods, but other organs and tissues, such as the alimentary canal, respiratory tract, uterus, prostate gland, etc., are also used. These cells provide monolayer cultures of usually large, polygonal, closely knit cells with round nuclei. Subculture can be accomplished by the trypsin method, although the number of possible serial passages varies with the tissue source and animal species used as donor.

Cultivation of the more specialized glandular epithelia with maintenance of functional activities is more difficult, but the success rate is steadily improving with the introduction of new methods and improved culture media. In most cases a gland contains multiple cell types, and problems arise in trying to isolate and identify the particular cells desired for culture. For many years this made the culture of pancreatic beta cells very difficult; however, methods have now been developed for the harvest of islets essentially free of other cellular elements (15). In the case of the pituitary gland, the types of cells appearing in the cultures can be partially controlled by careful dissection of the gland into its component parts. Continuous cell lines have been produced from pituitary tumors that continue to produce prolactin and growth hormone.

For many years liver cells were difficult to cultivate, but cultures of these cells are now easier to perform. In situ perfusion of the organ with enzymes has enhanced the preparation of viable cell suspensions for cultivation, and cells more prone to divide have been obtained from regenerating liver after partial hepatectomy. Improved culture media with more specific requirements for liver cells have been formulated. Although there are numerous studies on primary or short-term cultivation of hepatocytes, there are few established cell lines.

Cells from other glands (e.g., thyroid, parathyroid, adrenal, ovary, and testicle) have been cultured at least for limited periods of time with the production of specific hormones. This has been accomplished by using explant or organ cultures and by preparation of enzyme-dispersed cell suspensions. The fact that a cell produces a specific product aids greatly in the identification of the cells growing in the cultures.

Culture of endothelial cells lining the blood vessels and the heart has been greatly expanded in recent years, and these cells can now be cultured from arteries, veins, capillaries, and the heart. Further, several continuous lines of endothelial cells have become available. The cells from large arteries or veins are harvested by selecting a section of vessel, ligating one end, and filling it with collagenase. With repeated treatment the harvested collagenase solution will contain loosened endothelial cells which can be planted in culture (18, 22). A similar procedure involves everting the blood vessel onto a rod, thus exposing the endothelial lining, and repeatedly dipping or stirring the rod in a solution of collagenase. For the preparation of capillary endothelium, a tissue rich in microvascular material is selected, minced into small fragments, and homogenized or treated with collagenase. The resulting small fragments of capillary tissue are collected by filtration through nylon mesh. Further cell dispersion may be done with collagenase, or the fragments and aggregates may be planted into culture. Endothelial cells settle and attach to the culture vessel rapidly, and this can be used as a means of reducing the number of contaminating cells (fibroblasts, smooth-muscle cells) by early rinsing and refeeding of the cultures. Several growth factors when added to the medium have been reported to enhance the growth of endothelial cells. These include tumor cell-conditioned medium, thrombin, fibroblast growth factor, and endothelial cell growth supplement.

The culture of cells related to the immune system is essential to the new discipline of cellular immunology. The methods for obtaining and cultivating these cells were covered in Methods for Primary Culture (section 20.5). Numerous continuous cell lines of lymphocytes and macrophages are also available.

20.9 SOME SPECIFIC USES OF CELLS IN CULTURE

The uses and applications of cells in culture are increasing as more scientific information on growth regulation, differentiation, drug effects, function, and hormonal regulation is accumulated.

20.9.1 Effects of Chemical Compounds on Cell Proliferation

There are a variety of procedures for measuring the effects of compounds on cell proliferation. These include measurements of cell numbers by microscopic observation, use of automated equipment such as volume counters and flow cytometers, incorporation of isotopically labeled precursors into macromolecules (such as DNA, RNA, and proteins), and cell plating efficiencies. Quantitation methods similar to those used with microorganisms can be used (2, 3).

20.9.2 Genetic Studies

The use of mammalian cell cultures in genetic studies is expanding rapidly, prompted in part by the developments in molecular biology and by new techniques for measuring genetic changes in cells. Cell culture is playing a key role by permitting direct observations of the cells in question. Skin fibroblasts, peripheral blood leukocytes, and amniocentesis samples have all been used as sources of cells for these studies in humans. Cell banks with specific genetic markers have been established in the American Type Culture Collection and the Human Genetic Mutant Cell Respository. Methods for the growth of cells and specific procedures for identifying chromosomal abnormalities are described in publications from both institutions and in various tissue culture methodology handbooks. Genetic studies are being enhanced by rapid developments in hybridoma technology.

Other specific areas of research in which cell culture plays a major role are host-parasite relationships, virus studies, recombinant DNA studies, and growth regulation and differentiation. Differentiation has received considerable attention during the last several years due to advances in the development of hormonally defined media. The use of mammalian cell cultures in basic research and applied developmental situations is rapidly expanding.

20.10 CELL PRESERVATION AND RECOVERY

To ensure that cells are available for future use and to safeguard against accidental loss due to contamination or inadequate or improper culture conditions, cultures may be stored in liquid nitrogen. The viability of cells in liquid nitrogen is preserved for many years.

Preservation

1. Trypsinize a subconfluent population of rapidly growing cells, or, in the case of suspension cultures, centrifuge cells that are in log phase. Resuspend the pellet in cool complete growth medium (including serum) at a concentration of 2.5×10^6 to 4.0×10^6 cells per ml. Slowly add reagent-grade dimethyl sulfoxide or glycerol to this solution to achieve a final concentration of 5 to 10%.
2. Use a syringe fitted with an 18-gauge needle to transfer 1 ml of the cell suspension to a sterile glass ampoule, which is then flame sealed (plastic vials are also commercially available).
3. Place the ampoule in the vapor phase of a liquid nitrogen freezer for 30 min. Alternatively, place the ampoule in a Styrofoam box with sides and lid that are 5 to 10 cm thick. Place this box in a −70°C freezer for 2 h. Either of these procedures gives a slow cooling rate and eliminates the expense of controlled freezing rate apparatus.
4. Place the ampoule in commercially available racks, and store in a liquid nitrogen freezer.
5. After 24 h, check viability and sterility.

Recovery

Generally, the simplest method involving the fewest operations and the least handling is recommended for cell recovery from storage.

1. Remove an ampoule from the liquid nitrogen freezer. Gloves, face mask, and long-sleeved garments should be worn. There is a danger of ampoule explosion if liquid nitrogen has leaked into it.
2. Quickly thaw the contents of the ampoule by placing it in a 37°C water bath. Agitation increases the rate of thawing.
3. Cleanse the outside of the ampoule with 70% ethanol and allow it to dry.
4. Break off the end of the ampoule in a sterile towel, and carefully remove the contents with an 18-gauge needle and syringe.
5. Add sufficient complete growth medium to dilute the dimethyl sulfoxide to a concentration of less than 0.1%. If glycerol is used, the growth medium should be slowly added to the cell suspension and immediately centrifuged at $200 \times g$ for 5 min. Cell culture medium is used to resuspend the cells at the optimal cell density. Modification of this procedure can be made, but the object is to reduce the glycerol concentration in a stepwise fashion.
6. Incubate the cells at 37°C. After 24 h, replace the medium with fresh growth medium.

20.11 DETECTION OF CONTAMINATION

Check all cultures and each individual flask periodically for contamination by using a phase-contrast microscope. Periodic fluorescent staining and culturing for mycoplasma are also recommended.

Where antibiotics are used, maintain separate cultures in the absence of antibiotics. A quarantine of all new cell lines, media, serum, and enzymes before general use in the laboratory is recommended. Do not share the same bottles of medium, serum, or enzymes among different cell lines or technicians.

It is important to check the morphological characteristics of the cells periodically for contamination with other cell lines. Kits for isozyme determination are now commercially available to determine the presence or absence of enzymes characteristic to various species.

Mycoplasma

Mycoplasma contamination is a concern for every investigator who uses cultured cells. Data derived from cell cultures contaminated with mycoplasma should be considered invalid. Mycoplasma have been shown to have the following effects on cells: (i) interference with growth rates; (ii) cytopathic effects; (iii) depletion of essential nutrients from media; (iv) alteration of carbohydrate content of media; (v) inhibition of nucleic acid and protein synthesis; (vi) changes in enzyme patterns; (vii) chromosomal aberrations; (viii) modification and redistribution of cell antigens; (ix) modification of viral infectivity; (x) interference with the purification of cell organelles (mitochondria, membranes); and (xi) reduction of the oncogenicity of malignant cells.

The best way to avoid the effects of mycoplasma is to prevent cell contamination. Sources of infection are human, cattle, or swine sera. Therefore, only serum from high-quality suppliers should be used. Serum should be checked for the presence of mycoplasma by several of the assays currently available. Mouth pipetting must be avoided, and heat inactivation of serum should be considered. Several antibiotics have been reported to suppress the growth of mycoplasma on cells, but other studies suggest recurrence of the infections upon removal of the antibiotics. Other reports have recommended passage of the malignant cells in animals upon recovery of the cells. mycoplasma could not be detected.

Detection of infections

Microbiological and viral contamination of cells can be difficult to detect and assess. This is especially true of viral contamination since some viruses are species specific. While detection of viral and mycoplasma infections of mammalian cells is complex and requires special diagnostic methods, detection of ordinary bacterial and yeast infections usually requires only a good phase-contrast microscope. Some bacteria, however, adhere to cell surfaces or grow intracellularly, and special procedures must be used to isolate and detect these organisms. This is especially true for anaerobic and facultative organisms (see reference 3 for procedures for isolating, growing, and identifying these organisms).

Since mycoplasma infections of cells in culture affect many vital cell processes and properties and can remain unrecognized and undetected indefinitely, special procedures must be used periodically to determine their presence. The presence of mycoplasma usually is confirmed by demonstrating their characteristic growth on agar or by fluorescence microscopy.

Culture techniques for detection of mycoplasma infection

In our experience, broth and agar cultures have been the most sensitive means of detecting mycoplasma infections because such cultures have the advantage of rare-event detection with growth amplification. Since most mycoplasmas are bound to the cell surface, it is imperative that a cell suspension be prepared. Some mycoplasmas are shed and can be cultured from medium. Frequently, fragments of cells can be mistaken for mycoplasmas upon microscopic observation.

Mycoplasma broth

1. Combine 4.2 g of Difco PPLO broth and 20 ml of yeast extract (see below).
2. Suspend mixture in 140 ml of distilled water.
3. Autoclave at 15 lb/in^2 for 15 min.
4. Cool to 40°C and add 40 ml of mycoplasma-free horse serum.

Mycoplasma agar

1. Prepare broth as described above.
2. Add 2.4 g of Difco purified agar.
3. Autoclave.
4. Cool to 40°C and add 40 ml of mycoplasma-free horse serum.
5. Pipette 7.0 ml of the solution into 55-mm plates.
6. Invert and cool at 4°C.

Yeast extract preparation

1. Add 250 mg of dry Fleischmann's bakers' yeast to 1 liter of water.
2. Bring mixture to a boil with frequent stirring, and keep at a low boil for 10 min.
3. Remove from heat and let stand until yeast cells settle.
4. Centrifuge at 1,000 × *g* to remove most of the yeast cells.
5. Pipette 20 ml of the supernatant into separate vessels and freeze.

Mycoplasma growth and detection

1. Approximately 0.15 ml of sample is streaked onto the surface of duplicate agar plates and allowed to dry at room temperature.
2. One plate is incubated at 37°C in an anaerobic jar with a BBL GasPak (3). The other plate is incubated in an aerobic, moist atmosphere at 37°C.
3. At the same time 0.5 ml of sample is added to each of two 7-ml tubes of broth. One tube is incubated with the anaerobic plate, and the other tube is incubated with the aerobic plate.
4. Platings and passages are made at 5- and 10-day intervals with 0.5 ml transferred to fresh broth.
5. If no mycoplasma colonies develop on the original plates or after the subsequent two passages, a third passage is recommended.
6. Agar plates are examined by inverting them on the microscope stage and focusing through the agar. Low power (2.5 to 6.0×) is useful for locating the colonies. Mycoplasma colonies range in size (5 to 1,000 μm) and generally exhibit a characteristic "fried-egg" appearance. Higher-power objectives (10 to 20×) should be used to obtain greater detail.
7. Mycoplasma grow to high titer in broth culture without exhibiting turbidity. Microscope observation is necessary.

Nonculture techniques for detection of mycoplasma infection

Some strains of mycoplasma do not grow on standard agar plates or have become so adapted to growth in cell culture that their growth in other media is poor. A direct fluorescence stain may therefore be necessary. Cells should be grown on cover slips in Leighton tubes (2) or petri dishes, or on slides in petri dishes. The conditions for culture should be optimum for that particular cell, and the population should be grown to about 50% confluency.

Reagents

Hoechst stain 33258 (500 μg/ml) in Hanks balanced salt solution without phenol red or sodium bicarbonate.

Mounting medium (citric acid monohydrate, 4,662 mg; Na_2HPO_4, 8,247 mg; glycerol, 500 ml; water to 1,000 ml).

Procedure

1. Dilute the stain to 0.5 μg/ml at room temperature
2. Aspirate all of the medium from the culture.
3. Fix in Carnoys solution (glacial acetic acid-absolute methanol, 1:3) for 5 min. Remove the fixative and replace with fresh fixative. Let stand for 10 min.
4. Air dry the slide, cover it with stain solution, and hold for 30 min.
5. Remove the stain, and wash the slide three times in deionized
6. Air dry the slide and preserve in the mounting m

Examine the slide under a suitable fluorescent [illegible] excitation wave length, 360 nm; maximum emission wavelength, 490 to 500 nm). A negative [illegible] indicated by only nuclear staining. A culture positive for mycoplasma will be seen as a positive nuclear stain with the cytoplasm or cell membrane covered with small fluorescent material relatively uniform in size. Control cultures with and without deliberate mycoplasma infections should be used as controls.

5. Bacteria, yeast cells, and other organisms can be identified by their typical morphological characteristics.

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Short Report

Intra-rectal injection of tumour cells: a novel animal model of rectal cancer

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The purpose of this study was to develop an animal model of rectal cancer. Three murine-derived cell lines, B16 melanoma, CT26 and MCA38 colon carcinoma, as well as the human colon cancer cell line LS174T were injected into the submucosa of the mouse rectum. Subcutaneous CT26 anbd B16 tumours and intra-caecal CT26 tumours served as controls for tumourigenicity of the cell lines. B16 melanoma produced a locally aggressive rectal tumour as well as skin and para-aortic lymph node metastases. CT26 produced local tumour when injected intra-rectally and colon tumours and liver metastases when injected into the caecum. MCA38 and LS174T intra-rectal injections resulted in large rectal carcinomas without metastases. We believe that growth of a colon cancer cell line in the rectum approximates the human disease more closely than other models of colorectal cancer. We would expect that the model could similarly be utilized to assess the effects of novel adjuvant treatments for rectal cancer as well as in the study of the tumour biology of rectal cancer. *Surgical Oncology* 1991; 1: 251–256.

Keywords: **animal model, metastases, rectal cancer, tumourigenesis.**

INTRODUCTION

Rectal cancer is one of the most common internal malignancies of Western society and is one of the leading causes of cancer-related morbidity and mortality [1]. The primary definitive treatment modality is surgical excision. Adjuvant therapies have been studied extensively in recent years because of the high incidence of postoperative recurrences [2]. The lack of an appropriate animal model of rectal cancer has limited the ability to study the natural history of this disease and test new forms of therapy.

A number of experimental animal tumour models have been established to study various aspects of colon cancer. However, they were designed to test chemotherapeutic drugs and study tumour heterogeneity and metastatic potential of colon, not rectal cancer. These models include: rodent tumours induced by chemical carcinogens [3]; transplantable tumour lines derived from such lesions [4]; and xenografts of human colon carcinoma in nude mice [5]. Human colon tumour xenografts in nude mice are notable for their lack of metastatic behaviour when injected subcutaneously [6], and Morikata *et al.* demonstrated that human colon cancer cells lines must be injected directly into the caecal wall of nude

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mice to consistently develop metastases [7]. However, this model was difficult to establish because a laparotomy was required and was of limited relevance for the study of rectal cancer for anatomical and physiological reasons.

The purpose of this study was to develop an animal model of rectal cancer. Intra-rectal injection of tumour cells into the mouse rectum resulted in the formation of local tumours with a pattern of metastases consistent with distal rectal cancer in humans.

METHODS

Cell lines

The murine B16 F10 melanoma cell line was kindly provided by Dr R. S. Kerbel (Samuel Lunenfeld Research Institute). CT26 is a murine anaplastic colon carcinoma cell line originally induced by *N*-methyl-*N*-nitrosourethan [3]. The cell line was generously provided by Dr P. Frost (University of Texas Medical Center). MCA38 is a dimethylhydrazine-induced murine adenocarcinoma [8] obtained from Dr M. Goldrosen (Roswell Park). LS174T is a human colon adenocarcinoma cell line [9] provided by Dr J. Schlom (NIH). Cells were cultured in RPMI 1640 with 10% fetal calf serum and passaged when confluent.

Mice

Studies with B16 and MCA38 tumours were carried out with male C57BL/6J mice (Jackson Laboratories, Bar Harbor, Maine), aged 6–8 weeks. For CT26 experiments, female BALB/c mice (Jackson), aged 4–5 weeks were used. Female nude mice (Taconic, Germantown, NY) aged 6–8 weeks were used in the LS174T studies. All animals were housed in the research annex of the Samuel Lunenfeld Research Institute.

Technique

Intra-rectal injection. Animals were anaesthetized with Avertin, then placed in a supine position and the rectal mucosa prolapsed with digital pressure. Cells (in 50 μl PBS) were slowly injected submucosally with a 30-gauge needle.

Intra-caecal injection. A mid-line laparotomy was performed under general anaesthesia with Avertin. The caecum was exposed and exteriorized. Cells were injected into the apical lymphoid follicle of the caecum with a 30-gauge needle. The peritoneum was then closed in one layer and the skin was stapled with metal wound clips.

There were no postoperative mortality or complications with either technique. The mice were observed for 30 days, then sacrificed.

Histology

Mice were sacrificed by cervical dislocation and the rectum and anus resected in continuity then fixed in 10% neutral buffered formalin. A complete necropsy was also performed to identify metastases. The specimens were serially sectioned and processed for histological examination with haematoxylin and eosin.

RESULTS

Tumourigenicity of B16 melanoma: subcutaneous versus intra-rectal injection

Initial studies of intra-rectal tumour injection were carried out with B16 melanoma. Of 16 mice injected, 15 developed rectal tumours within one week. Para-aortic lymph node metastases were noted in 11 of these animals (Fig. 1, left). Skin metastases also developed in one mouse (Fig. 1, right). Extensive local pelvic invasion occurred in most cases however, bowel obstruction was not apparent. Another group of mice were injected subcutaneously with tumour cells to compare tumorigenicity of intra-rectal versus subcutaneous injections. Subcutaneous tumours developed in 4 of 5 mice but metastases did not occur. These data are summarized in Table 1.

Tumourigenicity of intra-rectal injection of murine and human colon cancer cell lines

Tumourigenicity of CT26 carcinoma was assessed by comparing intra-rectal, subcutaneous and intra-caecal implantations. As shown in Table 2, intra-rectal injections resulted in tumour take in 17 of 22 animals with para-aortic lymph node metastases in

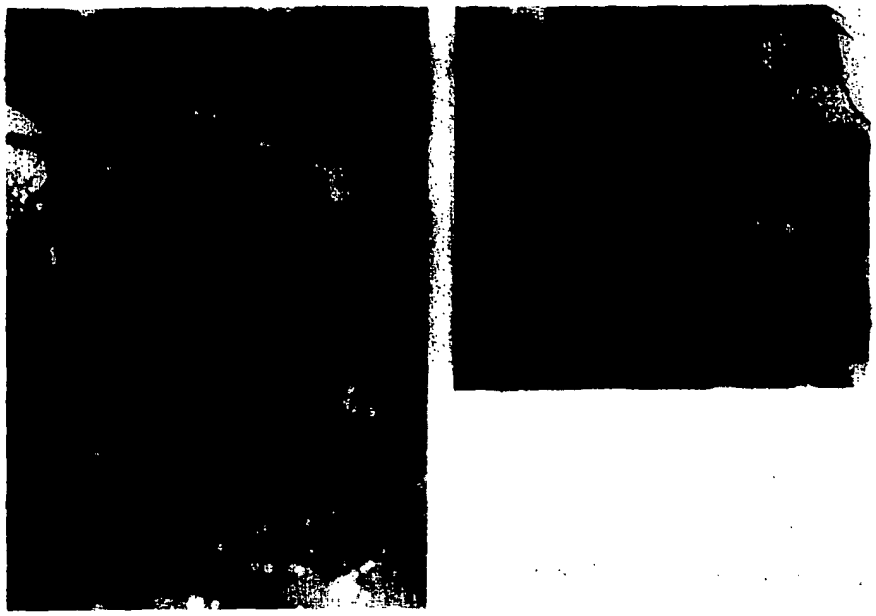

Figure 1. (Left) Para-aortic lymph node metastases (arrow) from B16 melanoma rectal tumour. (Right) Skin metastases (M) from intra-rectal implantation of B16 melanoma. The skin of the abdominal wall has been dissected off the peritoneum.

Table 1. Comparison of intra-rectal and subcutaneous injection of B16 melanoma

Injection site	B16 melanoma†	
	Rectum	Subcutaneous
Uptake	15/16	4/5
Time to first tumour (days)*	7	10
Para-aortic lymph node metastases	11/16‡	0/5

*Tumour size approximately 0.5 cm.
†2.5–7.5 × 10^5 cells injected.
‡One animal had skin metastases.

18%. A typical CT26 rectal tumour is shown in Fig. 2. Subcutaneous and intra-caecal injections resulted in tumour uptake in 5 of 5 and 4 of 5 animals, respectively. Liver metastases occurred in 2 of 5 animals following intra-caecal injection. Tumour uptake in the rectum occurred reliably with as low as 10^5 CT26 cells.

MCA38 murine adenocarcinoma reliably produced rectal tumours in C57BL/6J mice as did human adenocarcinoma LS174T when injected intra-rectally in nude mice (Table 2). In contrast to CT26, neither MCA38 nor LS174T tumours metastasized to para-aortic lymph nodes or the liver.

Table 2. Tumourigenicity of intra-rectal, subcutaneous and intra-caecal injection of CT26 and intra-rectal injection of MCA38 and LS174T colon cancer cell lines

Injection site	CT 26 carcinoma†			MCA38 carcinoma‡	LS174T carcinoma§
	Rectum	Subcutaneous	Caecum	Rectum	Rectum
Uptake	17/22	5/5	4/5	20/20	4/5
Time to first tumour (days)*	7	7	N/A	8	14
Para-aortic lymph node metastases	4/22	0/5	0/5¶	0/20	0/5

*Tumour size approximately 0.5 cm.
†0.5–1 × 10^6 cells injected.
‡2.0 × 10^5 cells injected.
§1.0 × 10^6 cells injected.
¶Liver metastases in 2 animals.

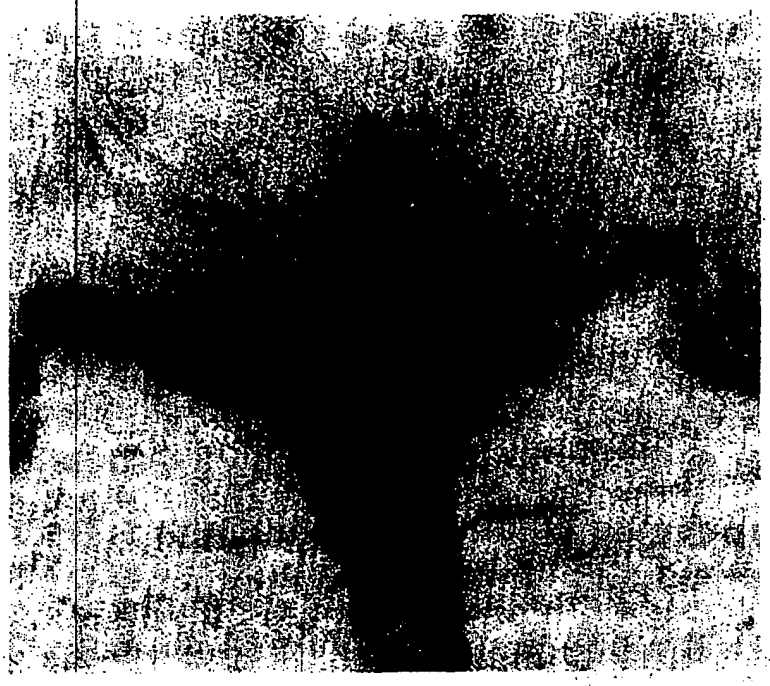

Figure 2. Anal swelling by rectal tumour produced by intra-rectal implantation of CT26 colon carcinoma. A cotton-tipped applicator is inserted in the anus to evert the tumour.

Histology

The majority of the tumour masses were perirectal with involvement of the muscularis propria and submucosa (Figs 3 and 4). Focal extension through the muscularis mucosa into the overlying mucosa was noted in several animals (Fig. 4).

DISCUSSION

The purpose of this study was to establish an experimental animal model of rectal cancer. The anatomy of the rectum of the mouse is similar to that of the human [10]. The rectum is rich in blood supply and its lower part is drained by both the systemic and the portal circulations. The pattern of lymph node drainage is similar to humans, and thus the model should be comparable to that of human rectal cancer which is characterized by aggressive local invasion and high metastatic potential. We have shown that submucosal injection of a single-cell suspension of cancer cells is capable of creating local tumours in the mouse rectum. We initially used B16 melanoma because it is known to be an aggressive tumour which grows rapidly and we wished to assess the feasibility of the technique. Indeed, we found that this cell line produced locally aggressive, invasive tumours within a week of injection. Furthermore, by comparing rectal with subcutaneous growth we showed that the tumour was also tumourigenic when injected into the rectum, and occasionally resulted in para-aortic lymph node metastases analogous to human rectal cancer.

We subsequently showed that a colon cancer cell line (CT26) is also capable of producing local rectal tumours. CT26 is a murine colorectal carcinoma that mimics the clinical behaviour of the human disease [11]. It has a rapid growth rate and a high metastatic potential when injected intra-caecally or systematically [11, 12]. The lack of liver metastases following intra-rectal injections may be due to the distal site of tumour injection in an area of the rectum drained primarily by the systemic and not the portal circulation. Moreover, the rectal tumours appeared to grow faster than caecal tumours and the mice were usually moribund at one month possibly before macroscopic liver metastases could develop. MCA38, another murine colon cancer cell line also reliably produced rectal tumours following intra-rectal injection but no liver or para-aortic metastases suggesting that it is inherently less aggressive than CT26. Intra-rectal injection of the human colon cancer cell line, LS174T results in tumours in 80% of nude mice but also does not produce metastases. We have been unable to produce lung 'metastases' with tail vein injections of LS174T in nude mice (unpublished observations) suggesting that this

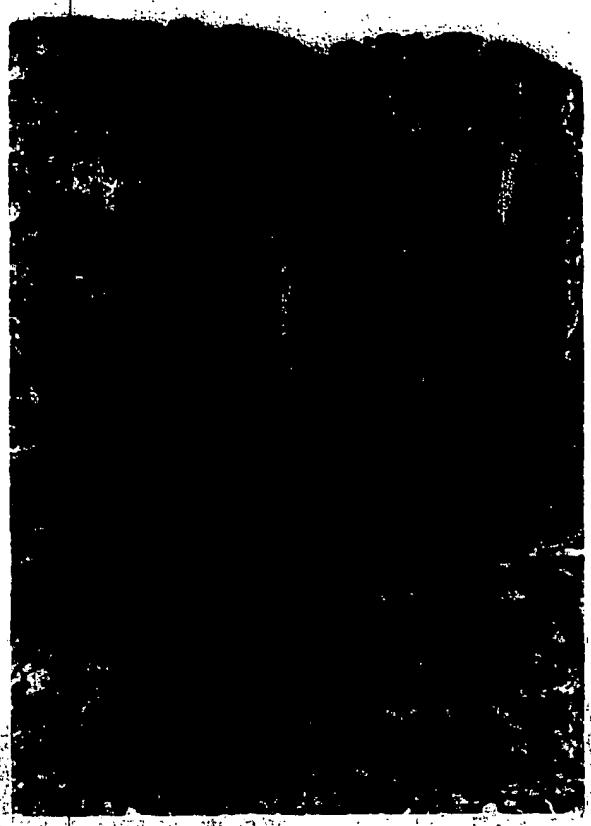

Figure 3. Rectal tumour induced by the local injection of CT26 colon carcinoma line. The tumour (T) is present in the submucosa. (Haematoxylin and eosin stain; original magnification × 250.)

Figure 4. Rectal tumour induced by the local injection of B6 mouse melanoma. The tumour (T) is present in the submucosa. Focally, the malignant cells breach the muscularis mucosa (arrow) and extend into the mucosa. (Haematoxylin and eosin stain; original magnification × 250.)

tumour xenograft is tumourigenic when injected into only a few sites such as subcutaneously or orthotopically in the rectum. It could be argued that para-aortic lymph node metastases following intra-rectal injection, as seen with B16 and CT26, occur only as a result of inadvertent injection into peri-rectal lymphatics. However, the lack of para-aortic lymph node metastases with some of the cell lines (MCA38 and LS174T) suggests that para-aortic lymph node tumours with B16 and CT26 represent true lymphatic dissemination from the primary rectal tumour.

The morbidity and mortality of rectal cancer that has failed conventional treatment can be profound. A rectal cancer model can be used in testing new therapeutic modalities. A number of investigators, including ourselves, are examining the role of various new treatment modalities such as photodynamic therapy. We are now utilizing the model established here to assess the effect of intra-tumour versus systemic delivery of the photosensitizer (haematoporphyrin) required in this form of therapy. Other potential uses for the model include studies of adoptive immunotherapy and monoclonal antibodies [13, 14]. Growth of a colon cancer cell line in the rectum approximates human disease more closely than other models of colorectal cancer such as subcutaneous injection of carcinogens or intracaecal injection of tumour cells. We would expect that the model could simply be utilized to assess the effects of other novel treatments.

ACKNOWLEDGEMENTS

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Helicobacter Colonization of Biopsy Specimens Cultured in Vitro Is Dependent on Both Mucosal Type and Bacterial Strain

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Meyer-Rosberg K, Berglindh T. *Helicobacter* colonization of biopsy specimens cultured in vitro is dependent on both mucosal type and bacterial strain. Scand J Gastroenterol 1996;31:434–441.

Background: Colonization by *Helicobacter pylori* is strictly tissue-specific. We have previously reported on an in vitro adhesion model for pig and human gastric mucosa, in which biopsy specimens were successfully infected and cultured for 72 h. The aim of this study was to compare *H. pylori* colonization of different mucosae and by different *Helicobacter* strains. *Methods:* Specimens from pig, rabbit, and rat antrum, pig urinary bladder, and pig duodenum were inoculated with two *H. pylori* strains and one *H. mustelae* strain. Four additional strains, including one mutant lacking flagella, were compared on pig antral specimens. *Results:* The viability of all mucosae was comparable at 48 h of culture. The percentage ahdering bacteria increased with time in all mucosae, reaching 17%, 11%, and 2% in pig, rabbit, and rat antral mucosa, 11% in pig bladder, and 3% in duodenum at 48 h. The type of *H. pylori* strain was a strong determinant for adhesion in pig antrum. Strain SVA40 had the highest adhesion; the mutant lacking flagella colonized very poorly. *H. mustelae* adhered to all types of mucosae in a more unspecific manner. *Conclusions:* On the basis of tissue viability, bacterial colonization, and adhesion, pig antral mucosa is clearly superior. *H. pylori* strains differ in their ability to adhere to and colonize cultured mucosa.

Key words: Gastric antrum; *Helicobacter mustelae*; *Helicobacter pylori*; in vitro; organ culture; pig; rabbit; rat; urinary bladder

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Helicobacter pylori is now recognized as the major pathogenic factor for the development of gastritis type B, duodenal ulcer (DU), and, possibly, gastric ulcer (GU) and gastric cancer (1–7). A histologically verifiable antral inflammation is always seen when the bacteria are present. Why some of these patients develop duodenal ulcers is unknown, as the vast majority of the population infected with *H. pylori* remains symptom-free (8) in spite of an often enhanced acid secretion. The presence of specific ulcerogenic strains of *H. pylori* would explain part of the pattern, as would the ability of the individual immune system to fight the infection.

H. pylori has developed several important properties for survival in the acidic and highly viscous environment of the stomach. These include a strong urease activity, which through the conversion of urea to ammonia is able to protect the bacteria from the low pH (9, 10), and a high mobility through the mucus, using the four to six polar flagella. The movements towards its final destination underneath the mucus layer are facilitated by the production of substances that decrease surface hydrophobicity (11) and/or degrade the glycoprotein structure of the mucus (12). Adhesion of *H. pylori* to mucus and to epithelial cells is thought to be important for establishment of infection and is thought to be mediated through specific recognition and receptor sites (13–15). A more detailed understanding of the mechanisms behind the adhesion to the gastric mucosa is, however, lacking.

Functional and relevant in vitro and in vivo models are of vital importance for the understanding of the pathogenic mechanisms of this bacterium. As far as is known at present, human gastric mucosa is the only naturally existing reservoir for *H. pylori*, and establishment of the infection in animals has been difficult (16, 17). Several animal models using closely related *Helicobacter* species that can induce inflammation in the gastric mucosa have been presented and are currently used to mimic *H. pylori* infection (18–20) but often do not have all the properties of the human pathogen. Animals susceptible to *H. pylori* infection include mice (21, 22), gnotobiotic beagles and pigs (23, 24), rhesus monkeys (25), and barrier-born pigs (26–28), of which several are expensive and cumbersome and require special housing. Several in vitro models describing adhesion of *H. pylori* have been presented, most of them being cancer cell lines such as HEp-2, KATO-III, HeLa, and mouse Y-1 adrenal cells (29–32) or freshly isolated human gastric epithelial cells (33, 34). However, saturated adhesion of *H. pylori* to these cells occurs very quickly (30 min), probably due to unspecific binding. Our previous reports on the gastric mucosa biopsy culture model for studies of *H. pylori* adhesion to pig and human biopsy specimens showed an increased adhesion over a much longer period of time—that is, 72 h (35, 36). The pattern of *H. pylori* adhesion to pig

Table I. Viability of in vitro-infected biopsy specimens

	24 h	48 h	72 h	*n*
Antrum				
Pig	12.9 ± 2.8 (100%)	11.6 ± 2.2 (89%)	11.3 ± 3.4 (87%)	28
Rabbit	14.1 ± 2.4 (100%)	12.6 ± 2.5 (91%)	8.5 ± 2.0* (60%)	5
Duodenum	[illegible]	[illegible]	[illegible]	[illegible]
Pig bladder	7.8 ± 2.0 (100%)	6.3 ± 1.8 (80%)	3.9 ± 1.8* (50%)	5

DPM/h x 10^3 per 3. Each 24-h period was measured separately.
* Significantly lower ^{3}H-leucine incorporation than at 24 h (percentage viability compared with 24 h). *n* = number of experiments.

antral specimens closely resembled that of human gastric mucosa (36), and electron micrographs show an intimate contact between *H. pylori* and microvilli/epithelial cells (37). Further, since the mucosal specimen is intact, with a functioning mucus production, we believe that this model more closely resembles the in vivo conditions than other in vitro models and would thus be more suitable for studies of *H. pylori* adhesion. These findings further support the use of pig also for in vivo studies, which have been done using both gnotobiotic and barrier-born pigs (23, 26–28).

The aims of this study were to in vitro 1) verify original observation from the pig; 2) show tissue/species specificity; 3) show tissue suitability; and 4) investigate bacterial/strain specificity.

MATERIALS AND METHODS

Pig mucosa

Mucosa from antrum, duodenum, and urinary bladder from the pig were used in this study. All parts were collected at the slaughterhouse within 20 min after slaughter. The stomach, duodenum, and urinary bladder were cut opened and rinsed with 0.9% NaCl. A piece of approximately 5 x 5 cm was cut out from the stomach (antrum), from the duodenum (5 cm below the pyloric sphincter), and from the bottom of the urinary bladder and immediately transported to the laboratory. Antral mucosa from 28 pigs, urinary bladder from 6 pigs, and duodenal mucosa from 6 pigs were included in this study. Mucosal biopsy specimens were prepared as previously described (35, 36). In brief, the mucosa was separated from the muscle layer, and 2-mm-wide specimens were punched out. Specimens were cultured with the mucosal side up on stainless steel grids with capillary contact to 1.5 ml culture medium in tissue culture dishes and incubated in air plus 7% CO_2 at 37°C. The total preparation time was approximately 2 h and 30 min. After 6 h in culture, at which point the mucosae had adhered to the grid, specimens were inoculated with *H. pylori* or *H. mustelae* (35, 36). The culture medium used was a modified Autrup's medium in which penicillin and streptomycin were replaced by nalidixic acid, 20 μg/ml; amphotericin (Fungizone®), 0.25 μg/ml; and Skirrow's antibiotic supplement, 0.4 ml/100 ml medium (Oxoid, Ltd., cat. no. SR-69). Specimens inoculated with *H. mustelae* were exposed to medium as above with the exclusion of nalidixic acid. All antibiotics except amphotericin were used in 50% concentrations when culturing specimens from bacteria-free rats, since the occurrence of contaminants was low in this tissue. The viability of the specimens was examined through the incorporation of ^{3}H-leucine. ^{3}H-labeled leucine was added to the culture media in a concentration of 2 μCi/ml (73 kBq), which together with the regular leucine resulted in a total concentration of 62 μg/ml. The number of *H. pylori* was followed up throughout the cultivation period with samples taken after 6 (time of inoculation), 24, 48, and 72 h.

Rat antral mucosa

Due to the insurmountable problem of bacterial contamination in the normal rat stomach, bacterium-free Sprague–Dawley rats weighing 150–200 g (kindly provided by Prof. T. Midvedt, Karolinska Institutet, Stockholm, Sweden) were used in this study. Rats ($n = 5$) were killed with 1.5 ml pentobarbital, 100 mg/ml intraperitoneally, after which the stomach was removed. The stomach was cut opened and gently rinsed with NaCl, and the antral part removed. Biopsy specimens were punched out as described above.

Rabbit antral mucosa

Four female New Zealand White rabbits (obtained from Estuna, Sweden) were given 1.5 ml pentobarbital/kg (Mebumal®) intravenously in a concentration of 30 mg/ml. The stomach was removed, cut open, and gently rinsed in NaCl, after which the antral part was cut out and specimens punched out (as above).

Strains

Two *H. pylori* strains were used in all types of mucosa. One was the type strain NCTC 11637, and the other was a strain named SVA40, isolated from a 42-year-old patient with a long history of peptic ulcer disease. SVA40 is a strain kept in our laboratory for several years and is known to establish infection in our barrier-born pig in vivo model (26–28). *H. mustelae* type strain CCUG 25715 was also tested on all types of mucosa. Two additional *H. pylori* strains were tested in specimens from pig antral mucosa only. These strains were freshly isolated and included AP2, a strain from a duodenal ulcer patient undergoing gastric surgery, and VBG4, from an

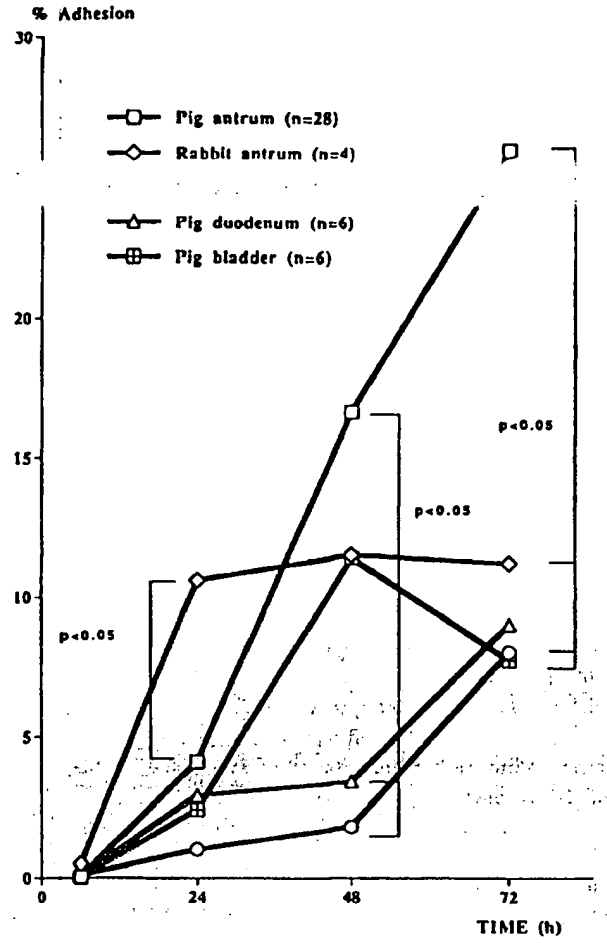

Fig. 1. Change in the percentage of viable *Helicobacter pylori* of strain SVA40 associated with different mucosae (after rinsing) during 72 h. Each point represents the mean value of the experiments of each mucosae (n). Four biopsy specimens were examined at each time point in each experiment. Statistically significant differences ($p < 0.05$) are noted by bars. Initially the rabbit antrum supported the adhesion best; at 72 h the pig antrum was superior to all other mucosae.

unsymptomatic patient undergoing gastric surgery owing to morbid obesity. In addition, strain 69Am, a genetically manipulated mutant lacking flagella, and its parent strain, 69A (kindly provided by Dr. Haas, Max-Planck-Institut, Tübingen, Germany), were tested in pig antral specimens. All strains were cultured on blood agar plates in a microaerophilic atmosphere during 48 h before inoculation (35, 36). The strains were passed a maximum of 10 times before inoculation.

Inoculation and detection of H. pylori

After 6 h in culture, biopsy specimens were inoculated with approximately 10^6 *H. pylori* or 10^6–10^7 *H. mustelae* per specimen with an inoculation loop. All strains were cultured on blood agar plates in a microaerophilic atmosphere for 48 h before inoculation. The number of bacteria was followed by viable count throughout the cultivation period with samples taken at 6, 24, 48, and 72 h of total incubation time. Viable count was performed after a vigorous rinsing procedure (five times in 30 ml NaCl on a Vortex mixer for 30 sec each time the specimen was ground in a mortar in 1 ml NaCl for 1 min. One hundred microlitres were spread out, direct and after dilution, on blood agar plates and incubated for a minimum of 3 days before colony-counting. By performing viable count also in the rinsing water, the total number of bacteria before and after rinsing could be calculated at each time point.

Our previous studies (35, 36) show that the inoculum varies between 10^6 and 10^7, but that this variation is not reflected in the amount of adhering bacteria (after rinsing) at the different time points.

An additional method to describe the adhesion is to express the increase in adhesion as AUC (area under the curve, percentage x hours; see Fig. 1 for comparison) which shows the trend over the entire cultivation period.

Statistics

All variables were studied in at least four specimens at each time point in each experiment (n). In general, 130–180 specimens were cultured in parallel. Owing to practical limitations, all strains could not be tested in all experiments (see tables and figures). Unpaired two-tailed t test was used for statistical evaluation: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; and NS = not significant.

RESULTS

Biopsy viability

The viability of specimens infected with *H. pylori*/*H. mustelae* is presented in Table I. The ^{3}H-leucine incorporation at 24 h was arbitrarily set at 100% and compared with the 48-h and 72-h values. As shown in Table I, no significant difference in viability was seen for the first 48 h. However, at 72 h only pig antral and pig duodenal mucosae showed a steady leucine uptake.

Adhesion to different mucosae

The adhesion of strain SVA40 to the different types of mucosal specimens up to 72 h of culture (66 h after inoculation) is presented in Fig. 1. Only a small fraction of bacteria was attached to the specimen at t = 0, ranging from 0.04% in pig antrum to 0.5% in rabbit antrum. The number of *H. pylori* adhering to the pig antral specimens increased almost linearly throughout the incubation time, having reached 26% at 72 h. In contrast, the adhesion to rabbit antral specimens increased sharply during the first 24 h (10.6% adhesion) but then remained at that level. The progression of adhesion to rat antral and pig duodenal mucosa was sluggish. Interestingly, the mucosa from the urinary bladder gave an

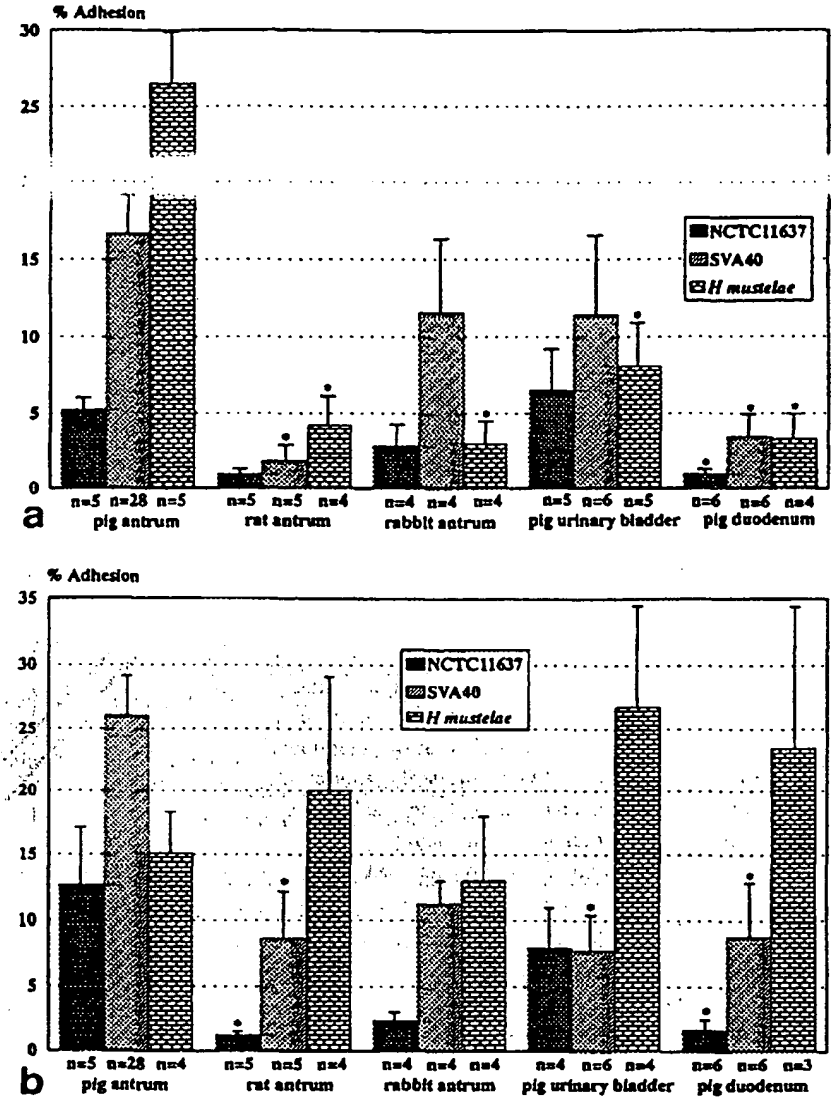

Fig. 2. Percentage viable bacteria of three different *Helicobacter* strains associated with rinsed mucosae of different types after a) 48 h and 2 b) 72 h of culture. Each point represents the mean ± SD of the experiments (n). Four biopsy specimens were analysed at each time point in each experiment. Statistical analysis is made by comparison with the values for the respective strain associated with pig antral mucosa. $^*p < 0.05$.

adhesion pattern very similar to that of pig antral mucosa for the first 48 h.

Specificity of strain

To compare the different strains (NCTC11637, SVA40, and *H. mustelae*) used in this study, the percentage adhesion of the strains to different types of mucosae at 48 h and 72 h is shown in Fig. 2a and b. AUC values for the different mucosae and strains are presented in Table III (up to 48 h).

Adhesion at 48 h. Of the two *H. pylori* strains, SVA40 showed the best adhesion to all mucosae, reaching 17% in the pig antrum (Fig. 2a). However, at the same time, *H. mustelae* displayed a 26% adhesion to this type of mucosa. This superiority was not general, since SVA40 gave higher values in rabbit antrum, pig duodenum, and pig bladder. Of the different mucosae, pig bladder best supported type strain NCTC 11637, with 7% adhesion, compared with 5% for pig antrum.

Adhesion at 72 h. The data should be interpreted with some caution since only pig antral and pig duodenal specimens showed a constant protein synthesis for 72 h. A decrease in protein synthesis might affect adhesion properties of the mucosae. SVA40 again showed the best adhesion properties within the *H. pylori* class, with the exception of urinary

Table II. Amount of viable (log 10) *Helicobacter pylori* (SVA40) in biopsy specimens. All mucosae are compared with pig antrum for statistical evaluation

	6 h	24 h	48 h	72 h	*n*
Antrum					
Pig	2.5 ± 0.6	4.2 ± 0.7	5.1 ± 0.8	5.4 ± 0.7	28
pig duodenum	2.7 ± 0.6	3.8 ± 0.8	4.2 ± 0.8*	4.4 ± 0.8**	6
pig bladder	2.2 ± 0.4	3.6 ± 0.5*	4.3 ± 0.5*	4.3 ± 0.3***	6

n = Number of experiments. Four biopsy specimens were examined at each time point; values represents the mean of all specimens. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; † = higher than pig antrum.

bladder, where NCTC 11637 was superior. *H. mustelae* showed at this time point high adhesion to all types of mucosae, with some preference for non-antral types.

Amount of H. pylori *in specimens*

The amount of bacteria actually adhering to the specimens gives information in addition to adhesion data. This is presented in Table II for SVA40 association with different mucosae. The amount of bacteria attached to the specimens is very low at the time of inoculation in all types of mucosa (160–630 *H. pylori*) with the exception of rabbit antral mucosa (4.0×10^3 viable *H. pylori*). Even though the amount of bacteria adhering to the specimens increased with incubation time in pig antrum, pig urinary bladder, and pig duodenal specimens, the amount of bacteria was much higher in pig antral specimens—that is, 2.7×10^5 at 72 h—as compared with 2.1×10^4 in urinary bladder and 2.8×10^4 in duodenal specimens. In rabbit antral specimens the number of viable *H. pylori* reached its maximum of 1.9×10^5 already at 24 h and did not increase further. The lowest amount of bacteria adhered to rat antral specimens, reaching only 3.8×10^3 and 1.7×10^4 after 48 and 72 h, respectively.

Adhesion of different strains to pig antral mucosa

The adhesion of the six different *H. pylori* strains and one *H. mustelae* is shown in Fig. 3. The adhesion of all strains was very low at 6 and 24 h but increased with cultivation time and showed a maximum at 72 h, with the exception of *H. mustelae*, which reached its maximal adhesion of 26% already after 48 h. It should be noted, however, that the same *amount* of *H. mustelae* adhered to the specimens at 72 h but that the amount of loosely attached bacteria had increased dramatically, resulting in a decrease in the percentage bacteria adhering. Strain SVA40 gave the highest percentage adhesion, reaching 26% at 72 h. As shown in Fig. 3, the pattern of adhesion was similar in strains VBG4, NCTC 11637, 69A, and AP2, in that the adhesion increased time-dependently throughout the cultivation time, but the degree of adhesion differed between strains. The strain with the lowest adhesion was the mutant lacking the gene coding for flagella, which resulted in a final adhesion of only 3%. The *H. pylori* strain lacking flagella adhered significantly less than its parent strain (9% at 72 h). The increase in adhesion was also calculated as AUC (Table III). Results confirmed that SVA40 adhesion was significantly superior to all other *H. pylori* strains. The strain lacking flagellae had a significantly lower AUC than its parent strain.

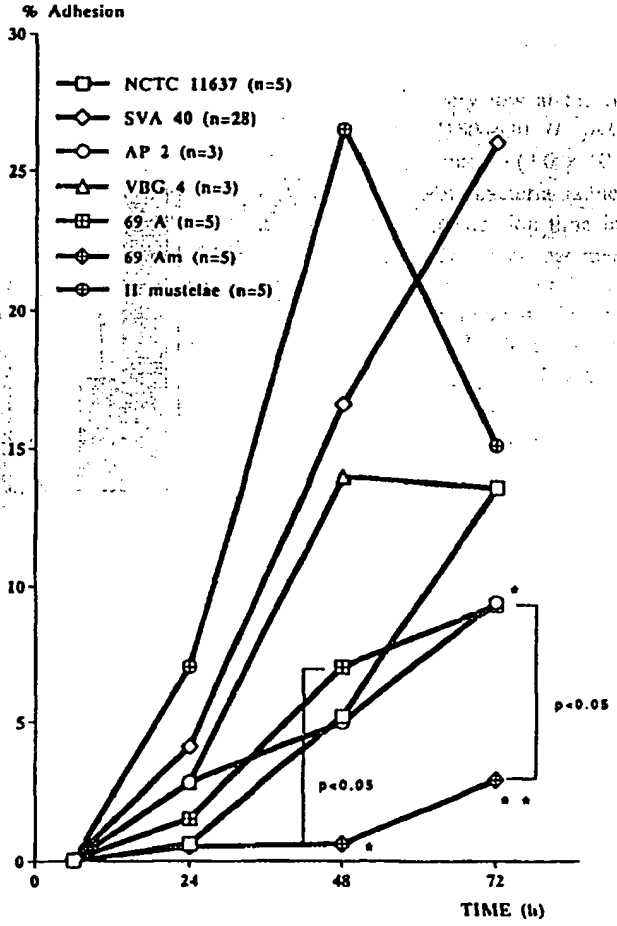

Fig. 3. Percentage viable bacteria associated with rinsed pig antrum after inoculation of seven different *Helicobacter* strains during 72 h. Each point represents the mean value of the experiments (n). Four biopsy specimens were analysed at each time point in each experiment. Statistical analysis (see Table III) showed that the mucosal association of SVA40 was superior to all other strains except *H. mustelae* and that the parent strain 69A had better adherence than the mutant-lacking flagella (69Am).

Table III. Area under the curve (AUC) of different strains on pig antrum at 6–72 h

Strain	AUC†	*n*
SVA40	790 ± 94	28
NCTC11637	300 ± 48***	5
[illegible]	[illegible]	5
69A	291 ± 94*	5
69Am	68 ± 27***‡	5
Helicobacter mustelae	962 ± 109^{NS}	5

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
† ±SEM.
‡ Paired, one-tailed *t* test.

DISCUSSION

The human gastric mucosa seems to be a unique matrix for survival and colonization of *H. pylori*. In fact, once established, a lifelong infection is likely to be the result. Such a 'perfect host' must have several specific features appreciated by *H. pylori*, such as nutrition, protection, and lack of competition (space). *H. pylori* must recognize both specific sites in the mucus and unique receptor sites on the epithelial cells. In this environment there might be typical chemotactic signals guiding the bacteria, and since a stressed *H. pylori* very rapidly undergoes morphologic transformation into a sturdy coccoid form, there must be warning receptors sensing the chemical environment. All these features will be highly species-specific, and thus *H. pylori* is adapted to conditions in the human gastric mucosa. Accordingly, it is difficult to establish *H. pylori* in a different environment, such as in different animal models. Minimum requirements for a suitable host of *H. pylori* must be that the general environment provides a growth matrix for the bacteria and that adhesion to mucus and epithelial cells can occur.

Detailed studies of bacterial colonization can only be performed in vitro, and for this purpose several models have been tried. Most reports concern transformed cancer cell lines with questionable relationship to the normal gastric mucosa. Saturable 'binding' occurs within 30 min, no mucus is present, and there does not seem to be any selectivity between different *H. pylori* strains (29–32). In the few reports dealing with freshly isolated gastric epithelial cells, similar findings are reported (33, 34). Although the isolated cell systems could be used to address some specific questions, the complexity of *H. pylori* colonization in most cases calls for a model that better resembles the in vivo situation.

This is the first study in which mucosal biopsy specimens from several animal species and from several organs have been cultured for up to 72 h and compared. Results from this study further verify our previous reports on the advantage of using pig antral specimens for studies of *H. pylori* adhesion in vitro (35). Our previous studies also included culture of human specimens, to which bacteria showed an adhesion pattern very similar to that in the pig (36). Since the pig is one of the few animals susceptible to *H. pylori* infection in vivo, it was of interest to compare this type of mucosa with gastric mucosa from other animals and with non-gastric mucosae.

In the evaluation of the data presented here it is important to consider that the adhesion to the mucosa in vivo depends on several dynamic factors, like immune system, acid secretion, [illegible]. This will lead to a lower relative adhesion, compared with the cultured in vitro mucosa, which lacks these factors. Thus in vitro we will directly measure colonization and 'receptor'-dependent properties.

Tissue specificity

The usefulness of the pig gastric mucosa as a matrix for *H. pylori* colonization was clearly illustrated in this study in that pig antral mucosa provided a superior milieu for growth and binding of *H. pylori* as compared with other antral mucosae and non-gastric mucosae. There are no reports that either the rabbit or the rat has been successfully infected with *H. pylori* in vivo. Although the initial adhesion to the rabbit antral mucosa was impressive, this mucosa showed a profound stickiness and a different time dependency. In humans the duodenum is not colonized by *H. pylori* unless gastric metaplasia exists. Our data indicate that pig duodenal mucosa might serve as a matrix, but the amount adhering is 10 times lower than for the pig antral mucosa. Since all pig tissue originated from slaughterhouse pigs, subjected to the stress associated with breeding and slaughter, the existence of gastric metaplasia in the duodenum is possible but was not determined.

In man the urinary bladder is a well-known site for adhesive bacterial infection. In fact, receptor sites for bacteria are probably better characterized in the bladder mucosa than in any other tissue. The finding that *H. pylori* could grow and progressively adhere to this mucosa was surprising and of potential future interest, since this model thus might be used for receptor mapping. Whether similar *H. pylori* binding sites are present in human bladder mucosa is unknown.

Tissue suitability

All mucosae had good viability for 48 h, but the only specimens having a consistent leucine incorporation throughout the cultivation period were pig antral and duodenal specimens. Unpublished data from this lab show that it is possible to culture pig antral specimens with maintained viability for up to 96 h.

Bacterial strain

In contrast to reported data of *H. pylori* adherence to isolated cells (34), the type of *H. pylori* strain was an important determinant for adhesion and colonization of antral specimens (Fig. 3). It is well known that strains differ in properties like urease production, motility, cytotoxicity, enzyme production, and their ability to agglutinate erythrocytes (38–41). To this list we can now add the ability to colonize and adhere to antral biopsy specimens. It is unknown

whether the above-listed properties are important for the association to the mucosa or whether the strains differ in their expression of adhesion molecules, chemotactic recognition, nutrient utilization, and so forth. Genetically manipulated strains of *H. pylori* will be available to test these hypotheses.

specimens were inoculated with a bacterial strain lacking the gene coding for the flagellae, a diminished adhesion (%) and a lower total number of bacteria colonizing the specimens were found. It is highly unlikely that this strain would be infectious in vivo owing to its inability to move away from the acid into the mucus. The latter reasoning is corroborated by studies in gnotobiotic piglets, in which a selected strain with low motility was unable to induce a sustained infection (42).

H. mustelae

In contrast to the human *Helicobacter* type, *H. mustelae* did not seem to be as fastidious, since it in general showed very good colonization abilities. This correlates to in vivo studies in the ferret showing that *H. mustelae* adheres deeper and closer to the epithelium than *H. pylori* in humans (43). In our biopsy model the total number of viable bacteria associated with the specimens was considerably higher than for *H. pylori*, reaching at least 10^5 already at 48 h in all types of mucosae except the urinary bladder. *H. mustelae* has to our knowledge been tried to a very limited extent in different animal models, whereas the non-adhering *H. felis* is successfully used to inoculate mice (20).

Conclusion

The present study has clearly shown that pig gastric specimens provide the best adherence and growth matrix for *H. pylori* compared with antral mucosa from other species. The pig antral mucosa is sturdy and shows good viability and a maintained mucus layer for 72 h. The pig mucosa seems to be able to support and distinguish between the adhesion properties of different *H. pylori* strains. It is very likely that the adherence capabilities are an important link with the virulence and pathogenicity of each strain. Two *H. pylori* strains where directly compared in all mucosae, and in all gastrointestinal-related tissue SVA40 was clearly superior to type strain NCTC 11637. However, these two strains showed the same adherence to pig bladder mucosa, a finding that might help to understand bacteria/receptor interactions.

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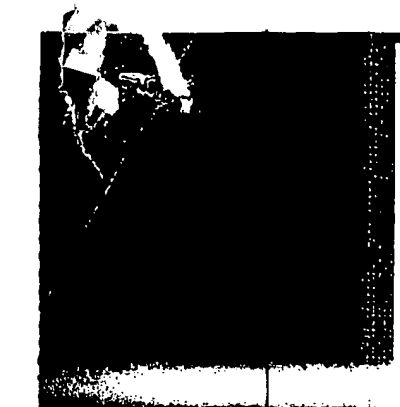

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ə-'kā-shəs\ adj (1658) : lacking the pow...
INEF FECTIVE — in-ef-fi-ca-cious-ly ...
-sē\ n [LL inefficacia, fr. L inefficax ...
- + efficac-, efficax efficacious] ...
e a desired effect
shən(t)-sē\ n, pl -cies (1749) 1 : the ...
t 2 : something that is inefficient ...
adj (1750) : not efficient: as a : not ...
r desired b : wasteful of time or energy
: : INCAPABLE, INCOMPETENT ⟨an ~ wo...
cient-ly adv
ga-lə-'ter-ē-ən\ adj (1940) : marked by ...
c standing
, adj (1748) : not elastic: as a : slow to ...
conditions b : INFLEXIBLE, UNYIELDING
i-s(ə-)tē ...
937) : a ...
particles changes into ...

(1938) : a scattering of particles as the ...
ich the total kinetic energy of the collid...

-gən(t)s\ n (1726) : lack of elegance
; [MF, fr. L inelegant-, inelegans, fr. ...
ca. 1570) : lacking in refinement, grace ...
y adv
ə-jə-bəl\ adj [F inéligible, fr. in- + ...
le: as a : not qualified for an office ...
er football rules to catch a forward pass
-'bi-lə-tē\ n — ineligible n
lə-kwənt\ adj (ca. 1530) : not eloquent

ək-tə-bəl\ adj [L ineluctabilis, fr. in- ...
x- + luctari to struggle, wrestle; akin ...
LOCK] (ca. 1623) : not to be avoided ...
— in-eluc-ta-bil-i-ty \-,lək-tə-'bi-lə-...
-blē\ adv
-də-bəl\ adj (1662) : INESCAPABLE
'nar-ə-bəl\ adj [ME, fr. MF, fr. L ...
xplain in detail, fr. e- + narrare ...
narrated : INDESCRIBABLE
MF inepte, fr. L ineptus, fr. in- + ...
s or aptitude : UNFIT 2 : lacking ...
uitable to the time, place, or occasion
ird degree 4 : generally incompetent
— in-ept-ly \-'nep(t)-lē\ adv —

nep-tə-,tüd, -,tyüd\ n [L ineptitudo ...
, or state of being inept; esp : ...
'kwä-lə-tē\ n [ME inequalite, fr. MF ...
qualitas, fr. inaequalis unequal ...
ne quality of being unequal or uneven ...
d disparity c : disparity of distribution ...
dition of being variable : CHANGEABLENESS
unequal 3 : a formal statement ...
ies usu. separated by a sign of inequality ...
spectively is less than, is greater than ...

-i-'ne-kwə-tə-bəl\ adj (1667) : ...
/ \-blē\ adv
e-kwə-tē\ n (1556) 1 : INJUSTICE ...
ce or unfairness
i-'nē-kwə-,valv\ also in-equi-...
e valves unequal in size and form

\i-ni-'ra-di-kə-bəl\ adj (1818) ...
erad-i-ca-bil-i-ty \-,ra-di-kə-...
-blē\ adv
i-'ner-ən(t)-sē\ n (ca. 1834) ...
the question of biblical ~ ...
adj [L inerrant-, inerrans, fr. ...
1837) : free from error
dj [L inert-, iners unskilled, idle ...
] (1647) 1 : lacking the power ...
SLUGGISH 3 : deficient in active ...
nticipated chemical or biological ...
— in-ert-ly adv — in-ert-ness n
8) : NOBLE GAS
-shə, -shē-ə\ n [NL, fr. L, ...
a property of matter by which ...
n in the same straight line ...
b : an analogous property ...
2 : indisposition to motion ...
r-tial \-shəl\ adj — in-er-tial-ly ...
nce n (ca. 1948) : guidance ...
s of self-contained automatic ...
rtial forces — called also inertial ...
\i-nə-'skā-pə-bəl\ adj (1792) ...
ed, or denied : INEVITABLE ...
\i-nə-'sen(t)-shəl\ adj (1677) ...
l : UNESSENTIAL — inessential n
e \(,)i-'nes-tə-mə-bəl\ adj [ME ...
aestimabilis estimable] (14c) ...
nputed ⟨storms caused ...
e measured or appreciated ...
y) — in-es-ti-ma-bly \-blē\ ...
\i-'ne-və-tə-bəl\ adj [ME ...
able] (14c) : incapable of being ...
ty \-,ne-və-tə-'bi-lə-tē\ n ...

, \-blē\ adv (15c) 1 : in ...
, it rained)
nig-'zakt\ adj [F, fr. L ...

... correct or tru... : INACCURATE ⟨an ~ translation⟩ 2 : not ... and careful ⟨an ~ thinker⟩ — in-ex-act-ly \-'zak(t)-lē\ adv ... -act-ness \-nəs\ n

...-ti-tude \(,)i-,nig-'zak-tə-,tüd, -,tyüd\ n [F, fr. inexact] (1782) ... of exactitude or precision 2 : an instance of inexactness

...-cel-sis \in-ik-'sel-səs also -'chel-\ adv [LL, on high] (1602) : in ... highest degree

...-sa-ble \,i-nik-'skyü-zə-bəl\ adj [ME, fr. L inexcusabilis, fr. in- + excusabilis excusable] (15c) : being without excuse or justification — ...-cus-able-ness n — in-ex-cus-ably \-blē\ adv

...-haust-ible \,i-nig-'zó-stə-bəl\ adj (1601) : not exhaustible: as a ... able of being used up ⟨~ riches⟩ b : incapable of being wea... worn out ⟨an ~ hiker⟩ — in-ex-haust-ibil-i-ty \-,zó-stə-'bi-lə-... — in-ex-haust-ible-ness \-'zó-stə-bəl-nəs\ n — in-ex-haust-... adv

...-tence \,i-nig-'zis-tən(t)s\ n (ca. 1623) : absence of existence

...

...-ra-ble \(,)i-'neks-rə-bəl, -'nek-sə-, -'neg-zə-rə-\ adj [L inexorabi-lis, fr. in- + exorabilis pliant, fr. exorare to prevail upon, fr. ex- + orare to speak, pray — more at ORATION] (1553) : not to be persuaded or moved by entreaty : RELENTLESS — in-ex-o-ra-bil-i-ty \(,)i-,neks-rə-'bi-lə-tē, ...-neg-zə-\ n — in-ex-o-ra-ble-ness \-'neks-rə-bəl-nəs, -'nek-... — in-ex-o-ra-bly \-blē\ adv

...-di-ence \,i-nik-'spē-dē-ən(t)s\ n (1608) : INEXPEDIENCY
...-di-en-cy \-ən(t)-sē\ n (1641) : the quality or fact of being ...
...-di-ent \-ənt\ adj (1608) : not expedient : INADVISABLE — in-...-ent-ly adv

...-sive \,i-nik-'spen(t)-siv\ adj (ca. 1846) : reasonable in price ... — in-ex-pen-sive-ly adv — in-ex-pen-sive-ness n

...-ence \,i-nik-'spir-ē-ən(t)s\ n [MF, fr. LL inexperientia, fr. L ... experientia experience] (1598) 1 : lack of practical experience ... knowledge of the ways of the world — in-ex-pe-ri-enced

...\(,)i-'nek-,spərt, ,i-nik-'\ adj [ME, fr. MF, fr. L inexpertus, fr. ... expert] (15c) : not expert : UNSKILLED — in-ex-pert... — in-ex-pert-ly \-'nek-,spərt-lē, ,i-nik-'\ adv — in-ex-...

...\(,)i-'nek-spē-ə-bəl\ adj [ME inexpyable, fr. L inexpiabi-lis, fr. ... expiare to expiate] (15c) 1 : not capable of being atoned for ... PLACABLE, UNAPPEASABLE — in-ex-pi-a-bly \-blē\ adv

...\,i-nik-'splā-nə-bəl\ adj (ca. 1923) : INEXPLICABLE
...\,i-nik-'spli-kə-bəl, (,)i-'nek-(,)spli-\ adj [ME, fr. MF, ... fr. in- + explicabilis explicable] (15c) : incapable of being ... interpreted, or accounted for — in-ex-pli-ca-bil-i-ty ... — in-ex-pli-ca-ble-ness ... — in-ex-pli-ca-bly \-blē\ adv

...-'spli-sət\ adj (ca. 1812) : not explicit
...-'spre-sə-bəl\ adj (1625) : not capable of being ex-... — in-ex-press-ibil-i-ty \-,spre-sə-'bi-lə-tē\ n ...-ble-ness \-'spre-sə-bəl-nəs\ n — in-ex-press-ibly

...-'spre-siv\ adj (1652) 1 archaic : INEXPRESSIBLE 2 ... or meaning ⟨an ~ face⟩ — in-ex-pres-sive-ly adv ...-ness n

...\,i-nik-'spəg-nə-bəl, -'spyü-nə-\ adj [ME in... fr. L inexpugnabilis, fr. in- + expugnare to take by storm ... pugnare to fight — more at PUNGENT] (15c) 1 : ... subdued or overthrown : IMPREGNABLE ⟨an ~ ...⟩ ... FIXED ⟨~ hatred⟩ — in-ex-pug-na-ble-ness n ... -bly \-blē\ adv

...-'spən-jə-bəl\ adj [in- + expunge] (1888) : inca-...

...-(t)-(,)sō\ adv [ML] (1826) : at full length ⟨the ...⟩

...-'stiŋ-gwi-shə-bəl, -'stiŋ-wi-\ adj (15c) : not ... QUENCHABLE ⟨an ~ flame⟩ ⟨an ~ longing⟩ — in-... adv

...-'strā-\ adv [L] (ca. 1530) : in extreme ... point of death

...\-kə-bəl, (,)i-'nek-(,)stri-\ adj [ME, fr. MF or ... fr. in- + extricabilis extricable] (15c) 1 : ... from which it is impossible to get free 2 a : ... untied ⟨an ~ knot⟩ b : not capa-... — in-ex-tri-ca-bil-i-ty \,i-nik-,stri-kə-'bi-lə-tē, ... — in-ex-tri-ca-bly \,i-nik-'stri-kə-blē, (,)i-'nek-...

... [ME, fr. ML infallibilis, fr. L in- + LL ...] incapable of error : UNERRING ⟨an ~ mem-...⟩ ... deceive, or disappoint : CERTAIN ⟨an ~ ...⟩ ... in defining doctrines touching faith or ... -bi-lə-tē\ n — in-fal-li-bly \-'fa-lə-...

...(1964) : moving under the influence of grav-... black hole) — in-fall \-,fȯl\ n

... [ME, fr. L infamis, fr. in- + fama fame] ... of the worst kind 2 : causing or bring-... convicted of an offense bringing infamy

...: evil reputation brought about by ... shocking, or brutal 2 a : an extreme and ... b : the state of being infamous

...(14c) 1 : early childhood 2 : a ... 3 : the legal status of an infant

... [ME enfant, fr. L infant-, infans, fr. ... speech, young, fr. in- + fant-, fans, ... (14c) 1 : a child in the first pe-... full age : MINOR

²infant adj (ca. ...) 1 : intended for young children 2 : being in an early stage of development 3 : of, relating to, or being in infancy

in-fan-ta \in-'fan-tə, -'fän-\ n [Sp & Pg, fem. of infante] (1593) : a daughter of a Spanish or Portuguese monarch

in-fan-te \in-'fan-tē, -'fän-(,)tā\ n [Sp & Pg, lit., infant, fr. L infant-, infans] (1555) : a younger son of a Spanish or Portuguese monarch

in-fan-ti-cide \in-'fan-tə-,sīd\ n [LL infanticidium, fr. L infant-, infans + -i- + -cidium -cide] (ca. 1656) 1 : the killing of an infant 2 [LL infanticida, fr. L infant-, infans + -i- + -cida -cide] : one who kills an infant — in-fan-ti-ci-dal \-,fan-tə-'sī-dᵊl\ adj

in-fan-tile \'in-fən-,tīl, -tᵊl, -,tēl, -(,)til\ adj (1696) 1 : of or relating to infants or infancy 2 : suitable to or characteristic of an infant; esp : very immature — in-fan-til-i-ty \,in-fən-'ti-lə-tē\ n

infantile paralysis n (1843) : POLIOMYELITIS

in-fan-til-ism \in-fən-,ti-,li-zəm, -tə-,li-; in-'fan-tᵊl-,i-\ n (1895) 1 : retention of childish physical, mental, or emotional qualities in adult life; esp : failure to attain sexual maturity 2 : an act or expression that ...

...

fan-til-i-za-tion \,in-fən-,ti-lə-'zā-shən, -fən-tᵊl-ə-; in-,fan-tᵊl-ə-\ n

in-fan-tine \'in-fən-,tīn, -,tēn\ adj (1603) : INFANTILE, CHILDISH

in-fan-try \'in-fən-trē\ n, pl -tries [MF & OIt; MF infanterie, fr. OIt infanteria, fr. infante boy, foot soldier, fr. L infant-, infans] (1579) 1 a : soldiers trained, armed, and equipped to fight on foot b : a branch of an army composed of these soldiers 2 : an infantry regiment or division

in-fan-try-man \-trē-mən\ n (1883) : an infantry soldier

infant school n (1824) Brit : a school for children aged five to seven or eight

in-farct \'in-,färkt, in-'\ n [L infarctus, pp. of infarcire to stuff, fr. in- + farcire to stuff] (1873) : an area of necrosis in a tissue or organ resulting from obstruction of the local circulation by a thrombus or embolus — in-farct-ed \in-'färk-təd\ adj — in-farc-tion \in-'färk-shən\ n

in-fare \'in-,far, -,fer\ n [ME, entrance, fr. OE infær, fr. in + fær way, fr. faran to go — more at FARE] (1595) chiefly dial : a reception for a newly married couple

¹in-fat-u-ate \in-'fa-chə-wət\ adj (15c) : being in an infatuated state or condition

²in-fat-u-ate \-,wāt\ vt -at-ed; -at-ing [L infatuatus, pp. of infatuare, fr. in- + fatuus fatuous] (1533) 1 : to cause to be foolish : deprive of sound judgment 2 : to inspire with a foolish or extravagant love or admiration — in-fat-u-a-tion \-,fa-chə-'wā-shən\ n

in-fau-na \'in-,fȯ-nə, -,fä-\ n [NL, fr. ²in- + fauna] (1914) : benthic fauna living in the substrate and esp. in a soft sea bottom — compare EPIFAUNA — in-fau-nal \-,fȯ-nᵊl, -,fä-\ adj

in-fea-si-ble \(,)in-'fē-zə-bəl\ adj (1533) : not feasible : IMPRACTICABLE — in-fea-si-bil-i-ty \-,fē-zə-'bi-lə-tē\ n

in-fect \in-'fekt\ vt [ME, fr. L infectus, pp. of inficere, fr. in- + facere to make, do — more at DO] (14c) 1 : to contaminate with a disease-producing substance or agent (as bacteria) 2 a : to communicate a pathogen or a disease to b of a pathogenic organism : to invade (an individual or organ) usu. by penetration c of a computer virus : to become transmitted and copied to (as a computer) 3 a : CONTAMINATE, CORRUPT ⟨the inflated writing that ~s such stories⟩ b : to work upon or seize upon so as to induce sympathy, belief, or support ⟨trying to ~ their salespeople with their enthusiasm⟩ — in-fec-tor \-'fek-tər\ n

in-fec-tion \in-'fek-shən\ n (14c) 1 : the act or result of affecting injuriously 2 : an infective agent or material contaminated with an infective agent 3 a : the state produced by the establishment of an infective agent in or on a suitable host b : a disease resulting from infection 4 : an act or process of infecting; also : the establishment of a pathogen in its host after invasion 5 : the communication of emotions or qualities through example or contact

in-fec-tious \-shəs\ adj (1542) 1 a : capable of causing infection b : communicable by infection — compare CONTAGIOUS 2 : that corrupts or contaminates 3 : spreading or capable of spreading rapidly to others ⟨their enthusiasm was ~⟩ ⟨an ~ grin⟩ — in-fec-tious-ly adv — in-fec-tious-ness n

infectious hepatitis n (ca. 1941) : HEPATITIS A

infectious mononucleosis n (1920) : an acute infectious disease associated with Epstein-Barr virus and characterized by fever, swelling of lymph nodes, and lymphocytosis

in-fec-tive \in-'fek-tiv\ adj (14c) 1 : producing or capable of producing infection 2 : affecting others : INFECTIOUS — in-fec-tiv-i-ty \(,)in-,fek-'ti-və-tē\ n

in-fe-lic-i-tous \,in-fi-'li-sə-təs\ adj (1835) : not appropriate in application or expression — in-fe-lic-i-tous-ly adv

in-fe-lic-i-ty \-sə-tē\ n, pl -ties [ME infelicite unhappiness, fr. L infelicitas, fr. infelic-, infelix unhappy, fr. in- + felic-, felix fruitful — more at FEMININE] (1617) 1 : the quality or state of being infelicitous 2 : something that is infelicitous

in-fer \in-'fər\ vb in-ferred; in-fer-ring [MF or L; MF inferer, fr. L inferre, lit., to carry or bring into, fr. in- + ferre to carry — more at BEAR] vt (1528) 1 : to derive as a conclusion from facts or premises ⟨we see smoke and ~ fire —L. A. White⟩ — compare IMPLY 2 : GUESS, SURMISE ⟨your letter ... allows me to ~ that you are as well as ever —O. W. Holmes †1935⟩ 3 a : to involve as a normal outcome of thought b : to point out : INDICATE ⟨this doth ~ the zeal I had to see him —Shak.⟩ 4 : SUGGEST, HINT ⟨another survey ... ~s that two-thirds of all present computer installations are not paying for themselves —H. R. Chellman⟩ ~ vi : to draw inferences ⟨men ... have observed, inferred, and reasoned ... to all kinds of results —John Dewey⟩ — in-fer-able also in-fer-ri-ble \in-'fər-ə-bəl\ adj — in-ferrer \-'fər-ər\ n

syn INFER, DEDUCE, CONCLUDE, JUDGE, GATHER mean to arrive at a men-...

I J K

L M

Studies of *Helicobacter pylori* in a Gastric Mucosa in vitro Animal Model

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Rosberg K, Hübinette R, Nygård G, Berglindh T, Rolfsen W. Studies of *Helicobacter pylori* in a gastric mucosa in vitro model. Scand J Gastroenterol 1991, 26, 43–48

A gastric mucosa in vitro model for studies of experimental *Helicobacter pylori* infections has been developed. Biopsy specimens were taken from pig gastric mucosa, infected with *H. pylori*, and cultured for up to 72 h. To determine the degree of *H. pylori* adhesion, specimens were vigorously rinsed by vortexing five times before measuring viable count and urease activity. The results showed that it is possible to culture pig gastric mucosa in vitro with maintained viability for at least 72 h. According to the viable count, the bacteria survived and multiplied during the whole culture period. The percentage viable *H. pylori* in the specimens after rinsing and the urease activity increased with time of culture. The results indicate that the bacteria in the gastric specimens were viable after 72 h and that there was a time-dependent increase in bacterial adhesion to the specimens. This in vitro gastric mucosa model promises to be an applicable and reproducible method, with high capacity, for both pathogenic and mechanistic studies of *H. pylori* infection.

Key words: Bacterial adhesion; *Campylobacter pylori*; gastric mucosa culture; *Helicobacter pylori*; mucosa in vitro model

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In spite of its recent discovery, *Helicobacter pylori* (formerly *Campylobacter pylori*) has been implicated as the common denominator for gastritis B and is closely associated with duodenal ulcer, gastric ulcer, and nonulcer dyspepsia (1–4).

The natural reservoir for *H. pylori* is the human gastric mucosa. Thus, a more detailed understanding of the pathogenicity and drug susceptibility of *H. pylori* in its native environment has been hampered by the lack of appropriate animal models. The attempts to establish animal models have to date been far from successful. Colonization of the gastric mucosa of animals such as rodents, cats, oppossums, rabbits, ferrets, and monkeys with the bacteria has been difficult to establish. The best current model of experimental *H. pylori* infection is the gnotobiotic and barrier-born pig (5–8). The pig is a functionally monogastric mammal with dietary habits and anatomic and physiologic characteristics similar to those of human gastric mucosa. However, these pig models are expensive and cumbersome to use.

A functioning tissue culture system would provide an excellent way to study *H. pylori* infection of pig gastric mucosa under controlled conditions (9–11). The aim of this study was to develop an applicable in vitro pig gastric mucosa model for studies of experimental *H. pylori* infection.

MATERIALS AND METHODS

Pig gastric mucosa

The pig stomach was collected from a slaughtered pig within 20 min after death. It was emptied and rinsed, and the antral part was cut out. The mucosa was separated from the underlying muscle

layer, and 2-mm-diameter biopsy specimens were punched out. Specimens were cultured in sterile, plastic culture dishes (Falcon Plastics, Los Angeles, Calif., USA) on a stainless steel grid placed in capillary contact with 1.5 ml culture media (12). Proper humidity was maintained by an outer well containing 4.5 ml 0.9% NaCl. Three specimens were placed on each grid, oriented with the mucosal side up, and incubated with air plus 7% CO_2 at 37°C in an incubator (Forma Scientific). A total of up to 150 specimens were cultured in parallel.

Biopsy culture media

The culture medium used was Autrup's medium (13, 14), in which the penicillin and streptomycin were replaced by nalidixic acid, 40 μg/ml; amphotericin (Fungizone®), 0.25 μg/ml; and Skirrow's antibiotic supplement in double recommended concentration (Oxoid, Ltd., cat. no. SR-69). [^{3}H]-labeled leucine (DuPont Nen Products, Boston, Mass., USA) in a concentration of 2 μCi/ml was added to the medium to measure the leucine incorporation. When the biopsy specimens were maintained for more than 24 h, the medium was changed every 24 h.

Biopsy viability test

To measure the [^{3}H]-leucine incorporation, samples were taken after 24, 48, and 72 h of cultivation. Three biopsy specimens from the same culture dish were dissolved in 1 ml tissue solubilizer (Soluene-350, Packard Instr.). Ten milliliters scintillation fluid (Hionic-Fluor, Packard Ind.) were added before samples were counted in a liquid scintillation counter (LKB Wallac 1214 Rackbeta).

Inoculation of H. pylori

After 6 h in culture, at which time the explants had attached to the grid, the specimens were inoculated with approximately 10^6 *H. pylori* per specimen with an inoculation loop.

H. pylori *strain*

The *H. pylori* strain used was NCTC 11637. Preliminary results are also shown for a new clinical *H. pylori* isolate taken from a 43-year-old patient with a long history of gastric ulcer. The strains were cultured on blood agar plates (Blood Agar Base, Oxoid), supplemented with 7% horse blood. Both isolates were cultured in a microaerophilic atmosphere (4% O_2, 7% CO_2, and 89% N_2) for 2 days before inoculation.

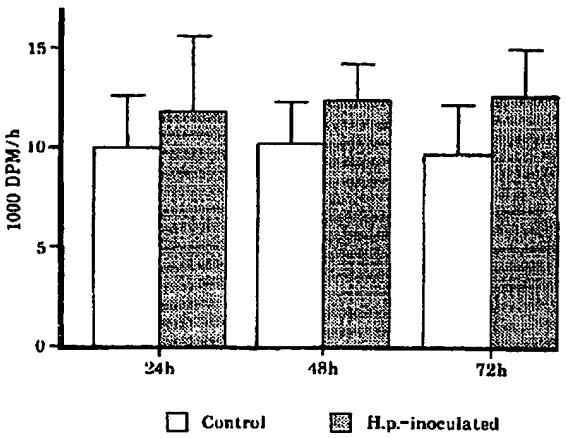

Fig. 1. [^{3}H]-Leucine incorporation (in 1000 dpm/h) in *Helicobacter pylori*-infected and noninfected biopsy specimens after a total of 24, 48, and 72 h. Values represent mean ± SD; four experiments with three to four culture dishes at each sampling time.

H. pylori *detection*

Biopsy samples were collected immediately after inoculation and after a total of 24, 48, and 72 h of cultivation (including the first 6 h). Noninfected specimens were used as controls for all variables except viable count. After cultivation each specimen was rinsed vigorously five times in 30 ml fresh 0.9% NaCl in a tube on a Vortex mixer for 30 sec, to remove loosely attached bacteria. The presence of *H. pylori* in the thoroughly washed specimen was studied by means of viable count and urease activity. Viable counts were also performed on the total rinsing water.

Viable count

The washed biopsy specimen was homogenized in a sterile mortar in 1 ml 0.9% NaCl for 1 min. One hundred microliters were spread out, direct and after dilution, on blood agar plates and incubated for a minimum of 3 days before colony counting. Since the rinsing water was tested in

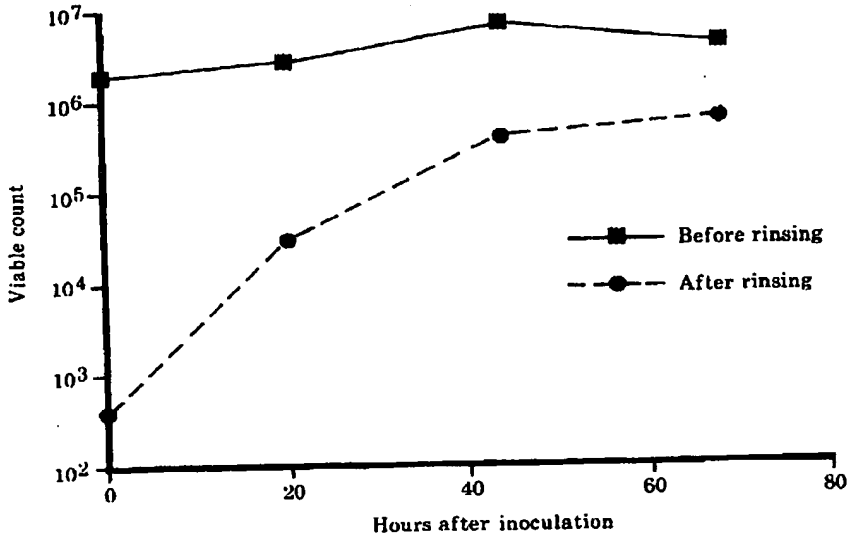

Fig. 2. Viable count of *Helicobacter pylori* before and after rinsing, after 24, 48, and 72 h of cultivation (0 h represents the time of inoculation, 6 h of cultivation time). Each point represents the geometric mean values of three biopsy specimens.

the same manner, the number of *H. pylori* in each specimen before rinsing could be calculated. Viable bacteria in the biopsy culture media was also determined as described above.

Urease activity

Each specimen was put into 1 ml freshly prepared urea solution consisting of 2% urea and 0.001% phenol red, and the change of color due to increase in pH was measured in a spectrophotometer at 559 nm (Shimadzu UV visible rec. spec. UV 160, Lambda Instr. AB). The absorbance was measured after 1, 10, and 60 min at room temperature.

Student's *t* test was used for statistical analysis.

RESULTS

Viability test

As shown in Fig. 1, the [^{3}H]-leucine incorporation did not differ either between control and *H. pylori*-infected specimens or among the different cultivation times. The tendency to a higher incorporation in the *H. pylori*-infected specimens was not significant.

Viable count

Fig. 2 shows the content of viable bacteria in specimens before and after rinsing with *H. pylori* isolate NCTC 11637. The number of viable bacteria before rinsing stayed stable throughout the whole cultivation period. However, the amount of *H. pylori* remaining in the specimen after rinsing increased with time. According to Fig. 2, at the time of inoculation only a few hundred *H. pylori* were attached to the specimen after rinsing. After 24 h of culture the number of viable *H. pylori* in the specimen had risen to 10^4–10^5 and increased further to 10^5–10^6 after 48 and 72 h. As can be seen in Fig. 2, the difference between the number of viable *H. pylori* before and after rinsing decreased with time. The number of viable *H. pylori* in the culture media for every 24-h period was approximately 10^8 after 24 h and stayed at that level throughout the experiment. The reproducibility of this technique is shown in Fig. 3, where results from three different experiments on NCTC 11637 are presented. This figure shows the percentage *H. pylori* left in the specimen after rinsing. Inset in Fig. 3 are the preliminary results from the adhesion of a freshly obtained *H. pylori* isolate. This isolate bound to

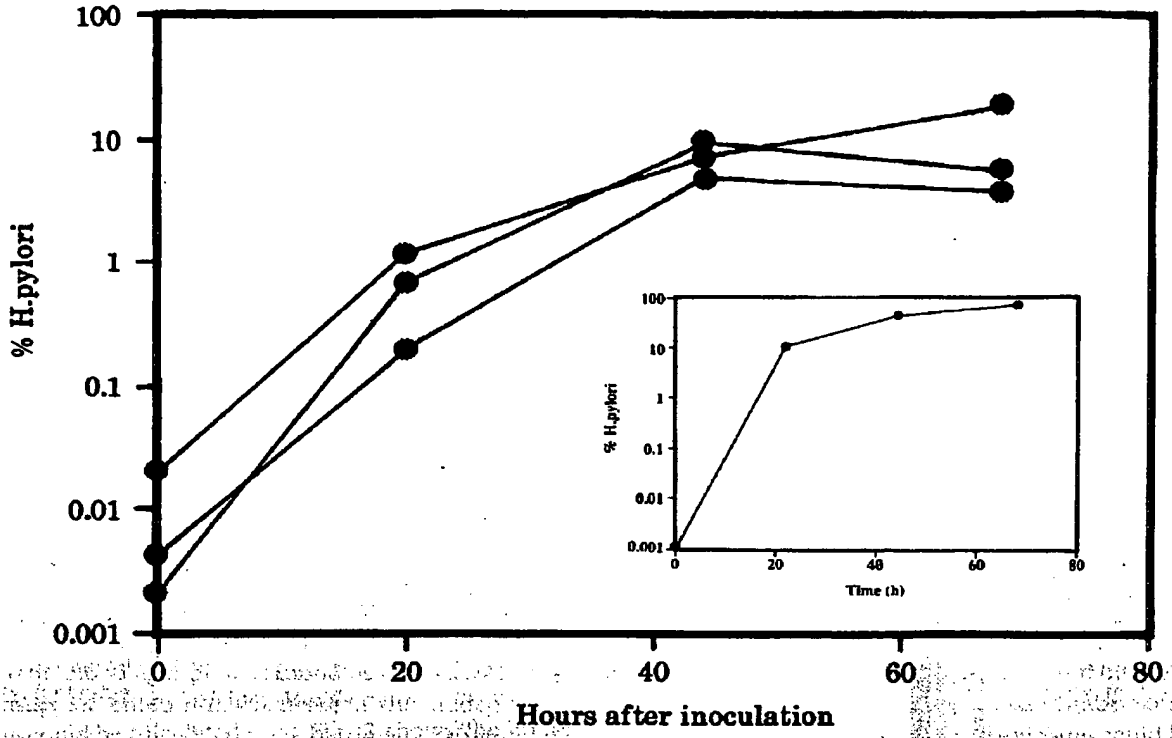

Fig. 3. Viable count of *Helicobacter pylori* in rinsed biopsy specimens after 24, 48, and 72 h of cultivation (0 h represents the time of inoculation, 6 h of cultivation time). The values represent the percentage of the total amount of *H. pylori* left in the specimen after rinsing. Each point represents the mean values of three specimens. Inset: In a single experiment the adhesive property of a new isolate freshly obtained from gastric mucosa was tested. This isolate bound to specimens with the same kinetics as previously seen, but the total amount bound was considerably higher—that is, 70% at 72 h. In the same experiment the regular NCTC 11637 isolate gave a 8% adhesion.

specimens with the same kinetics as previously seen, but the amount of bound *H. pylori* was considerably higher.

Urease activity

The urease activity was measured in NCTC 11637-inoculated biopsy specimens. The activity measured in the rinsed specimens after 24, 48, and 72 h of cultivation is shown in Fig. 4. After 1 min in the urea solution the 48-h- and 72-h-inoculated specimens showed a significant difference both between each other ($p < 0.01$) and compared with the 24-h-inoculated specimens ($p < 0.05$). All inoculated specimens measured after 10 min indicated a higher value than the controls but did not result in a significant difference between control and 24-h-inoculated specimens. Both 48 h and 72 h of culture gave a significant difference ($p < 0.01$) compared with 24 h. When the specimen was kept in the urea solution for 60 min, the absorbance maximum was reached regardless of cultivation time of the inoculated specimens.

DISCUSSION

Appropriate animal models for studies of experimental *H. pylori* infection are necessary if the field of *H. pylori* research is to continue its forward movement. In this study an in vitro pig gastric mucosa model for *H. pylori* research has been developed.

Our results indicate that it is possible to culture pig gastric mucosa in vitro with maintained

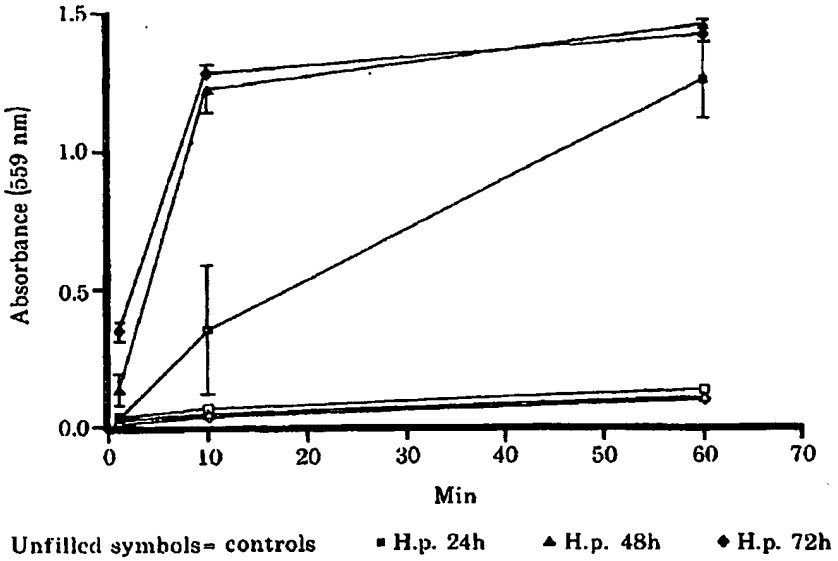

Fig. 4. The urease activity measured as change in phenol red absorbance in rinsed biopsy specimens after 24, 48, and 72 h. The measurements are made at 1, 10, and 60 min. Each point represents mean ± SD of three specimens. 1 min: 24-h-infected specimens did not differ from the controls; 48-h-infected differed from both 24-h-infected and controls ($p < 0.05$); 72-h-infected differed from 48 h ($p < 0.01$). 10 min: 24-h-infected did not differ statistically from controls; both 48-h- and 72-h-infected differed from 24-h-infected and controls ($p < 0.01$).

viability for at least 72 h, and this was not affected by the presence of *H. pylori*. The trend towards an increased protein synthesis in the infected specimens might be explained by an uptake of leucine by the bacteria themselves.

Results from the viable counts clearly indicate that *H. pylori* survived 66 h (72–6 h) in a biopsy specimen. The number of viable *H. pylori* in the specimen before rinsing was quite stable over time. More importantly, the viable count after rinsing increased with time, indicating that a large number of bacteria become more firmly attached with increasing incubation time. This might be due to *H. pylori* being able to penetrate the mucus and possibly adhere to the epithelial cells. In NCTC 11637-inoculated specimens the percentage *H. pylori* remaining after rinsing after 72 h was approximately 10%, while preliminary data from a freshly prepared isolate shows approximately 70% remaining bacteria in the specimen. These results indicate that different *H. pylori* isolates bind to the specimens with the same kinetics but have different adhesive properties. The urease studies confirm the viable count results, and our results suggest penetration and/or adherence of *H. pylori* to the gastric mucosa.

In conclusion, the in vitro pig gastric mucosa model is an applicable method for studies of experimental *H. pylori* infections. The method can be a potential model for mechanistic studies of *H. pylori* and for assessment of the efficacy of different therapeutic agents. The model has a high capacity—a very large number of specimens and variables can be studied at the same time—and the method is reproducible. Furthermore, this model is closer to the physiologic conditions than most other in vitro models—that is, isolated cells—since some basic physiologic conditions are preserved.

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HANDBOOK OF

Microbiological Media

By
RONALD M. ATLAS

Edited by
LAWRENCE C. PARKS

CRC Press
Boca Raton Ann Arbor London Tokyo

Supplement Solution:
Composition per liter:

Glucose	100.0g
L-Cysteine·HCl	25.9g
L-Glutamine	10.0g
L-Cystine	1.1g
Adenine	1.0g
Nicotinamide adenine dinucleotide	0.25g
Vitamin B_{12}	0.1g
Thiamine pyrophosphate	0.1g
Guanine·HCl	0.03g
$Fe(NO_3)_3 \cdot 6H_2O$	0.02g
p-Aminobenzoic acid	0.013g
Thiamine·HCl	3.0mg

Source: The supplement solution IsoVitaleX® enrichment is available from BBL Microbiology Laboratories. This enrichment may be replaced by supplement VX from Difco Laboratories.

Preparation of Supplement Solution: Add components to distilled/deionized water and bring volume to 1.0L. Mix thoroughly. Filter sterilize.

Preparation of Medium: To 500.0mL of sterile GC agar base aseptically add 500.0mL of sterile hemoglobin solution at 45°–50°C. Mix thoroughly. Aseptically add 10.0mL sterile supplement solution. Mix thoroughly. Pour into sterile Petri dishes or distribute into sterile tubes.

Use: For the isolation and cultivation of fastidious bacteria, especially *Neisseria* and *Haemophilus* species. For the cultivation and maintenance of *Branhamella catarrhalis, Campylocbacter pylori, Eikenella corrodens, Helicobacter pylori, Moraxella nonliquefaciens, Morococcus cerebrosis, Oligella ureolytica. O. urethralis, Pasteurella volantium, Proteus mirabilis,* and *Taylorella equigenitalis.*

GC Agar (GC Medium) (ATCC Medium 1351)

Composition per 1.0L:

GC agar base	950.0mL
Blood, defibrinated	50.0mL

pH 7.2 ± 0.2 at 25°C

GC Agar Base:
Composition per liter:

Agar	10.0g
Pancreatic digest of casein	7.5g
Peptic digest of animal tissue	7.5g
NaCl	5.0g
K_2HPO_4	4.0g
Cornstarch	1.0g
KH_2PO_4	1.0g

Source: GC agar base is available as a premixed powder from BBL Microbiology Systems. This base may be replaced by GC medium base available from Difco Laboratories.

Preparation of GC Agar Base: Add components to distilled/deionized water and bring volume to 1.0L. Mix thoroughly. Gently heat until boiling. Autoclave for 15 min at 15 psi pressure–121°C. Cool to 75°–80°C.

Preparation of Medium: To 950.0mL of sterile GC agar base aseptically add 50.0mL sterile defibrinated blood with thorough mixing and maintain at 75°–80°C for 15–20 min until the medium is chocolatized. Pour into sterile Petri dishes or distribute into sterile tubes.

Use: For the isolation and cultivation of fastidious bacteria, especially *Neisseria* and *Haemophilus* species. For the cultivation and maintenance of *Branhamella catarrhalis, Campylocbacter pylori, Eikenella corrodens, Helicobacter pylori, Moraxella nonliquefaciens, Morococcus cerebrosis, Oligella ureolytica. O. urethralis, Pasteurella volantium, Proteus mirabilis,* and *Taylorella equigenitalis.*

GC Agar with Ampicillin

Composition per 1020mL:

GC agar base, 2x	500.0mL
Hemoglobin solution	500.0mL
Supplement solution	10.0mL
Ampicillin solution	10.0mL

pH 7.2 ± 0.2 at 25°C

GC Agar Base, 2X:
Composition 500mL:

Agar	10.0g
Pancreatic digest of casein	7.5g
Peptic digest of animal tissue	7.5g
NaCl	5.0g
K_2HPO_4	4.0g
Cornstarch	1.0g
KH_2PO_4	1.0g

Source: GC agar base is available as a premixed powder from BBL Microbiology Systems. This base may be replaced by GC medium base available from Difco Laboratories.

Preparation of GC Agar Base, 2X: Add components to distilled/deionized water and bring volume to 500.0mL. Mix thoroughly. Gently heat until boiling. Autoclave for 15 min at 15 psi pressure–121°C. Cool to 45°–50°C.

Hemoglobin Solution:
Composition 500mL:

Bovine hemoglobin	10.0g